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Award Number DAMD17 - 98-1-8133

TITLE: Toward a New Chemotherapy for Breast Cancer: Structural and Functional Mechanism of the Retinoid Receptors Addressed by a Novel Computer Approach

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REPORT DATE: May 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | May 1999 | Annual (1 May 98 - 30 Apr 99) |

**4. TITLE AND SUBTITLE**
Toward a New Chemotherapy for Breast Cancer: Structural and Functional Mechanism of the Retinoid Receptors Addressed by a Novel Computer Approach

**5. FUNDING NUMBERS**
DAMD17-98-1-8133

**6. AUTHOR(S)**
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**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
New York University Medical Center
New York, New York 10016

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

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**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 words)*

Retinoids are known to be potent inhibitors of the proliferation of breast cancer cells, and recent observations have showed that they could be efficient molecular tools for breast cancer therapy. Our purpose is to help understand and control the activity of retinoid receptors, in order to design a new generation of drugs, more selective and efficacious, for breast cancer prevention and treatment. From a model of the active form of the Retinoid X Receptor (RXR), we could derive the determinants for retinoid receptor selectivity. We were then able to predict the mode of interaction between the Retinoic Acid Receptor (RAR) or RXR and known ligands. We also modeled the interaction between RAR or RXR and NRIF3, a novel nuclear receptor coactivator. Eventualy, we discovered three novel agonists and two novel antagonists of RAR, which could lead to new drugs against breast cancer.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
|---|---|
| Breast Cancer – Retinoid Receptors – Structure – Computer Modeling – Drug Design – Agonist – Antagonists | 90 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | limited |

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18 298-102

USAPPC V1.00

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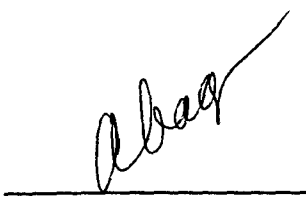

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## Introduction

Retinoids, natural and synthetic derivatives of vitamin A, are known to be potent inhibitors of the proliferation of breast cancer cells (Budd et al, Fitzgerald et al., Giannini et al.). Retinoids, as well as Tamoxifen, the molecular compound of choice used in hormone-dependent breast cancer chemotherapy, regulate the transcriptional activation pathway mediated by specific nuclear hormone receptors. The recent observation that they could be efficient molecular tools for breast cancer treatment has lead to an explosion of information concerning the mechanism of action of these molecules (Moras and Gronemeyer for review). It is now clear that understanding and controlling the activity of retinoid receptors will enable the design of a new generation of drugs, more selective and efficacious, for breast cancer prevention and treatment. Our goal is to use computer modeling to identify the structural determinants of retinoids which confer them their receptor selectivity and their agonist or antagonist activity, and rationally design molecules with various affinity for the Retinoic Acid Receptors (RARs) and the Retinoid X Receptors (RXRs).

## Body

Research accomplishment associated with each task outlined in the statement of work:

Task 1: Identify the structural determinants which confer a ligand RXR or RAR selectivity

1-Predict the structure of RAR-Ligand Binding Domain (RAR-LBD) bound to 9-cis Retinoic Acid (9-cis RA):

We used the structure of RAR-LBD bound to All-Trans Retinoic Acid (All-Trans RA) to dock 9-cis RA into the ligand binding pocket of the receptor. Both the ligand and the receptor side-chains were flexible in our improved docking algorithm (Totrov and Abagyan), and the resulting model could be used for the next step.

2-Model the structure of RXR-LBD bound to 9-cis RA:

We used the crystal structure of unliganded RXR and the result from step 1 to model by homology the RXR-LBD/9-cis RA complex (figure 1). We came to the unexpected finding that the overall shape of the RXR binding pocket is shorter and bulkier than the RAR ligand binding pocket. This result shows that All-Trans RA is RAR selective because it is too long for the RXR binding pocket.

3-Dock RXR- and RAR-selective ligands into the receptors pocket:

9-cis RA (RAR and RXR specific), All-Trans RA (RAR selective), BMS961 (RAR selective) and Targretin (RXR selective) were docked into the binding pocket of RAR-LBD (crystal structure) and RXR-LBD (model), and the complexes were relaxed according to an extensive energy minimization procedure. The Van der Waals

interactions (Gvw) between the receptors and ligands were then calculated. Figure 2 shows that, for each of the eight complexes, the calculated Gvw was negative when the ligand experimentally did bind the receptor, and was positive when the ligand experimentally did not bind the receptor. This perfect correlation between the models and the experiments was an encouraging indication of the validity of our approach.

4-Identify the functional groups of the ligands responsible for their affinity either for RXR or for RAR:

The results from step 3 show that the overall shape of the binding pocket determines the specificity of interaction, more than the network of hydrogen-bond donors and acceptors. Indeed, the fact that the Van der Waals term correlates with the affinity of the ligands for the receptors indicates that steric parameters are the critical receptor selectivity. Figure 2 shows for each ligand which is/are the functional group(s) sticking out of the binding pocket and responsible for Van der Waals clash.

Task 2: Identify the structural properties a ligand must satisfy to be an agonist

The original strategy was to model the interaction between RXR and the TATA binding protein (TBP), which is believed to be a coactivator. Important data regarding the identification, activity and structure of nuclear receptor coactivators has been published since (L. Freeman for review), which enabled us to refine our approach: upon binding of an agonist, the LBD undergoes a conformational change, which generates a hydrophobic pocket at its surface. This hydrophobic pocket is in turn the binding site for a conserved LxxLL motif of coactivator proteins. On the other hand, antagonists are believed to disrupt the formation of the hydrophobic pocket, and thus prevent complexation of coactivators to the receptor.

We collaborated with Dr Herbert Samuel's lab, at NYU Medical Center, to identify a novel coactivator, NRIF3, mediating functional specificity of nuclear hormone receptors. This novel protein coactivates RXR but not RAR. The manuscript presenting this work was submitted for publication, but is not published yet; it is therefore confidential, and appended to this report. Our modeling suggests that NRIF3 binds to liganded receptors through an LxxIL module contained within its C-terminal domain.

Task 3: Design rationally and test novel drugs against breast cancer

1-Use our model to screen libraires of compounds and select ligands with desired structural characteristics:

We plan to design novel agonists and antagonists for both RAR and RXR, and test them. We first applied our virtual screening algorithm to find RAR agonists and antagonists. Our approach to find antagonists was very innovative: we modeled the receptor in its inactive conformation, using as a template the crystal structure of the estrogen receptor bound to Tamoxifen, and used the resulting model to discover antagonists. The second confidential manuscript appended to this report describes more in detail this work.

2-Test the ligand candidates *in vitro*

Activity tests were performed and showed that we discovered three novel RAR agonists and two novel RAR specific antagonists. Some of the compounds discovered are very different from any RAR ligand described so far and could lead to novel drugs against breast cancer. The manuscript appended to this report is not published yet and these results are confidential.

3-Test these ligands *in vivo* for their efficacy against rat mammary tumor:

These tests remain to be done.

## Key research accomplishments

-We built a model of the active conformation of RXR

-We determined the structural differences between the RAR and RXR binding pockets, responsible for ligand selectivity.

-We modeled the interaction between known ligands and RAR or RXR and observed a very good correlation between our models and biochemical data.

-We modeled the interaction between RAR or RXR and NRIF3, a novel nuclear receptor coactivator.

-We discovered novel RAR agonists.

-We discovered novel RAR antagonists.

## Reportable outcomes

-Manuscript 1, confidential, submitted to *Molecular and Cellular Biology:*
NRIF3 is novel co-activator mediating functional specificity of nuclear hormone receptors.
D. Li, V. Desai-Yajnik, E. Lo, M. Schapira, R. Abagyan, and H. Samuels

-Manuscript 2, confidential, to be submitted to *Nature Medicine:*
Rational design of novel Nuclear Hormone Receptors
M. Schapira, B. Raaka, H. Samuels, and R. Abagyan

## Conclusions

During the first year of our efforts, we have already accomplished many of the tasks outlined in the approved Statement of Work. Many structural models were built, which showed very good correlation with available biochemical data, and shed some light on the specificity of interaction between the retinoid receptors and their ligands.

Our approach regarding the structural properties a ligand must satisfy to be an agonist was modified in view of important data recently published, and we could build a model which explains how, upon binding of an agonist to the receptor, a novel protein can activate selectively RXR.

We also were able to develop a strategy to rationally design antagonists, as well as agonists, for RAR. The activity of these novel molecules were confirmed *in vitro*.

"So what" section:

The relevance of our work was outlined by the discovery of novel agonists and antagonists of RAR. These molecules need now to be further optimized and their activity further characterized. We also need to conduct the same approach on the model of RXR that we built.

Our effort is therefore already contributing to a better understanding and control of the mechanism of retinoid receptors, and hopefully will enable the design of a new generation of drugs, more selective and efficacious, for breast cancer prevention and treatment.

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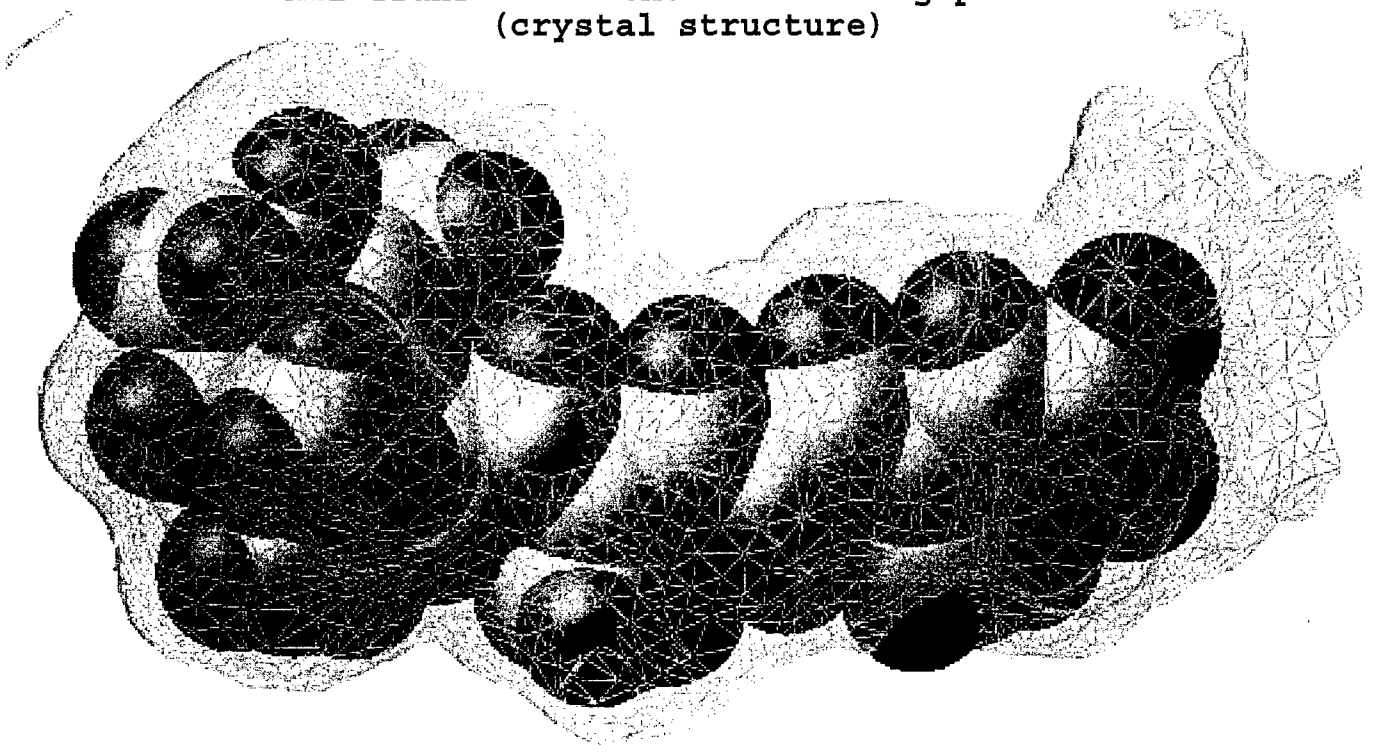
# Appendices

All-Trans RA in the RAR binding pocket
(crystal structure)



9-cis RA in the RXR binding pocket
(Model)

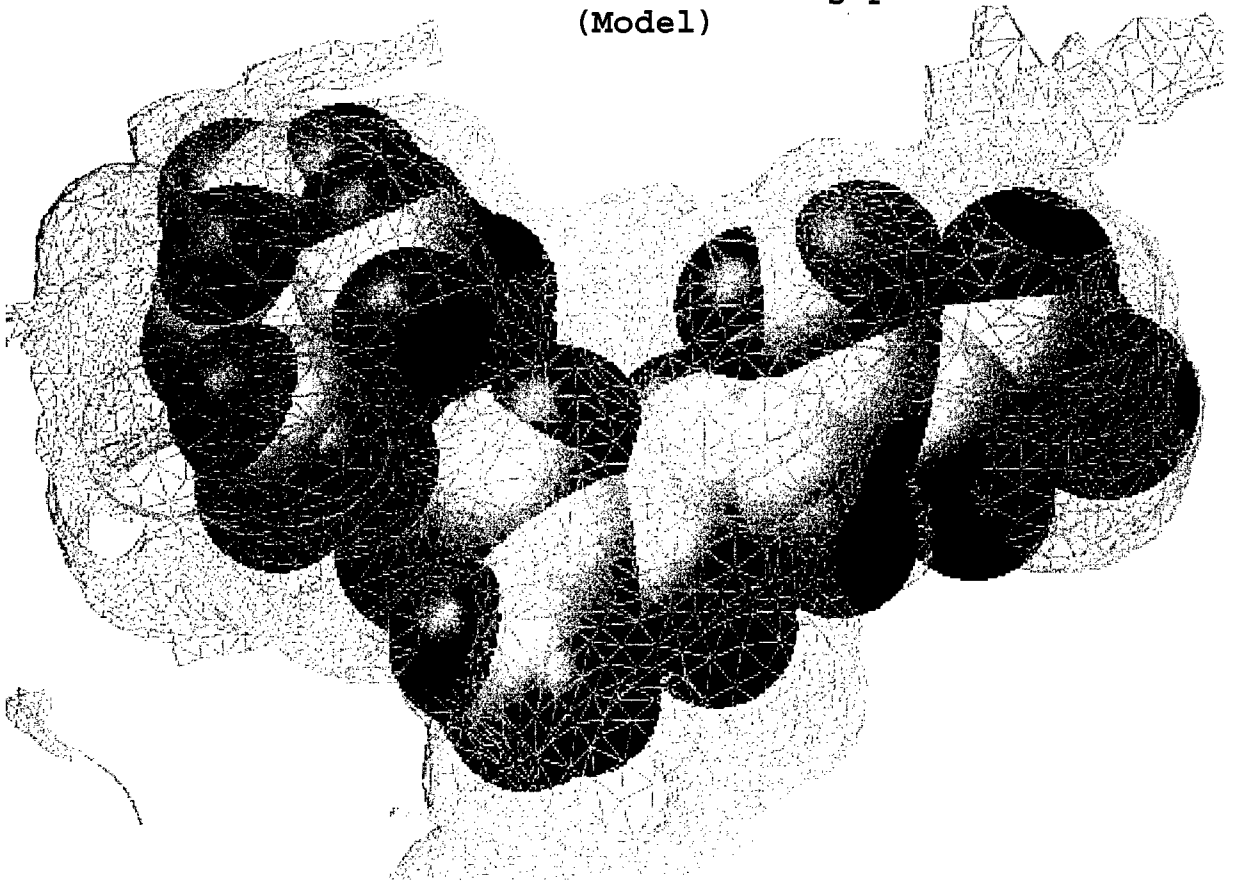
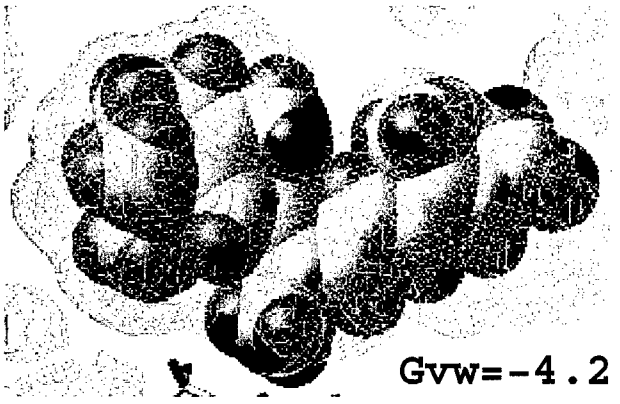
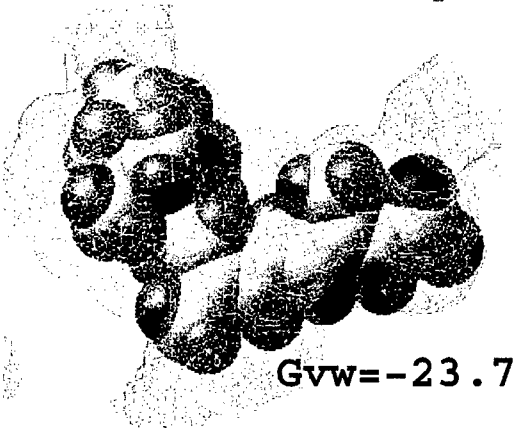


**Figure 1**: Crystal structure and model of retinoids in the binding pocket of the receptors. The Van der Waals boundary of the receptor is yellow.

RAR / 9cis RA complex

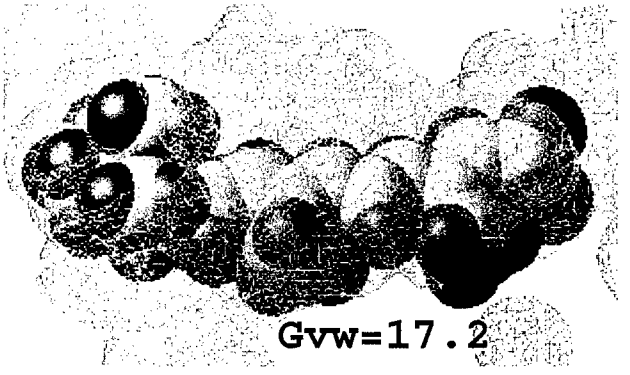



RXR / 9cis RA complex



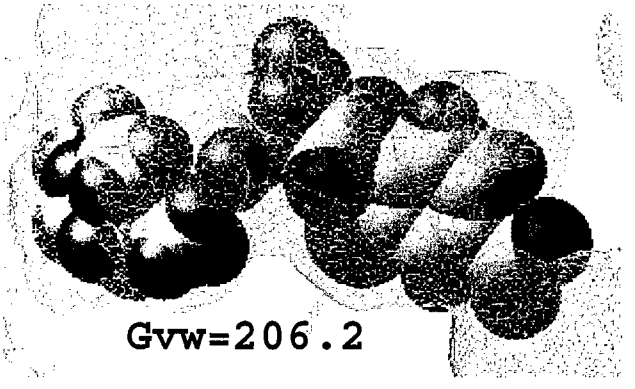

RAR / All-Trans RA complex


RXR / All-Trans RA complex

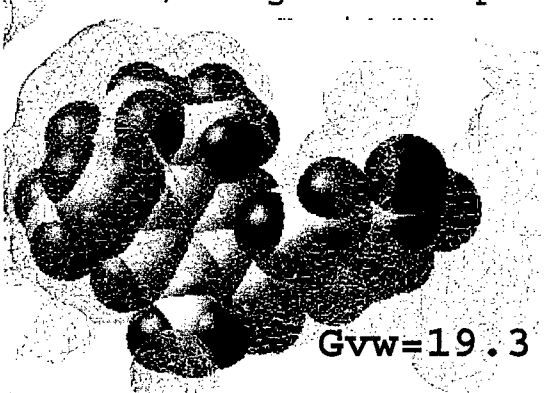



RAR / BMS961 complex


RXR / BMS961 complex




RAR / Targretin complex




RXR / Targretin complex

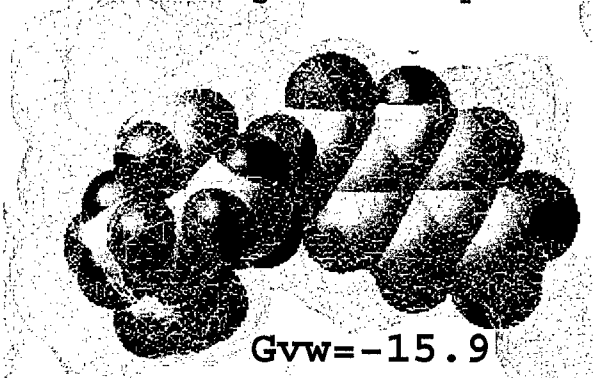



**Figure 2**: Known ligands docked into RAR and RXR. Complexes indicated in green are stable, in red are unstable (biochemical experiment). Gvw = calculated Van der Waals interaction (in kcal/mol)

# NRIF3 is a novel co-activator mediating functional specificity of nuclear hormone receptors

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**Running head: Co-activator and receptor specificity**

*Key words*: NRIF3; nuclear receptors; specificity; co-activators; transcription; hormone actions

Abstract

Many nuclear receptors are capable of recognizing similar DNA elements. The molecular event(s) underlying the functional specificity of these receptors (in regulating the expression of their native target genes) is a very important question that remains poorly understood. Here we report the cloning and analysis of a novel nuclear receptor co-activator (designated as NRIF3) that exhibits a distinct receptor specificity. Fluorescence microscopy shows that NRIF3 localizes to the cell nucleus. Yeast two-hybrid and / or *in vitro* binding assays indicate that NRIF3 specifically interacts with TR (thyroid hormone receptor) and RXR (retinoid X receptor) in a ligand-dependent fashion, but does not bind to RAR (retinoic acid receptor), VDR (vitamin D receptor), PR (progesterone receptor), GR (glucocorticoid receptor), or ER (estrogen receptor). Functional studies show that NRIF3 significantly potentiates TR- and RXR-mediated transactivation *in vivo* while little effect is observed for other examined nuclear receptors. Domain and mutagenesis analyses indicate that a novel C-terminal domain in NRIF3 plays an essential role in its specific interaction with liganded TR and RXR, while the N-terminal LxxLL motif plays a minor role in allowing optimum interaction. Computer modeling suggests that NRIF3 binds to liganded receptors through an LxxIL module contained within its C-terminal domain, where the combination of hydrophobic and electrostatic interactions generate receptor specificity. The identification of a co-activator with such a unique receptor specificity provides new insight into the molecular mechanism(s) of receptor-mediated transcriptional activation as well as the functional specificity of nuclear receptors.

## Introduction

Nuclear hormone receptors are ligand-regulated transcription factors that play diverse roles in cell growth, differentiation, development, and homeostasis. The nuclear receptor superfamily has been divided into two sub-families: the steroid receptor family and the thyroid hormone / retinoid (nonsteroid) receptor family (for a review, see Mangelsdorf et al. 1995). The steroid receptor family includes receptors for glucocorticoids (GR), mineralcorticoids (MR), progestins (PR), androgens (AR) and estrogens (ERs) (Mangelsdorf, et al. 1995). The nonsteroid receptor family includes receptors for thyroid hormones (TRs), retinoids (RARs and RXRs), 1,25-$(OH)_2$ vitamin D (VDR), prostanoids (PPARs) as well as many orphan receptors whose ligands (if any) remain to be defined (for reviews, see Mangelsdorf and Evans 1995; Mangelsdorf, et al. 1995). Members of the nuclear receptor superfamily share common structural and functional motifs. Nevertheless, an important difference exists between the two sub-families. Steroid receptors primarily act as homodimers by binding to their cognate palindromic hormone response elements (HREs) (Umesono and Evans 1989; Umesono et al. 1991). In contrast, members of the nonsteroid receptor family can bind to DNA as monomers, homodimers, and heterodimers (Umesono, et al. 1991; Forman et al. 1992). Their corresponding HREs are also complex, and can be organized as direct repeats, inverted repeats, and everted repeats (Mangelsdorf and Evans 1995). Therefore, the combination of heterodimerization and HRE complexity provides the potential to generate enormous diversity in receptor-mediated regulation of target gene expression.

Structural and functional studies indicate that the ligand binding domain (LBD) of many members of the thyroid hormone / retinoid receptor family harbors diverse functions. In addition to ligand binding, the LBD also plays roles in mediating receptor dimerization, hormone-dependent transactivation, and in the case of TR and RAR, ligand-relieved gene silencing (Nagpal et al. 1993; Qi et al. 1995). The carboxyl-terminal helix of the LBD has been implicated in playing an important role in ligand-dependent conformational changes and transactivation (Barettino et al. 1994; Durand et al. 1994; Baniahmad et al. 1995; Leng et al. 1995). Although it has been suggested that an activation function (AF-2) resides in this C-terminal helix, recent studies indicate

that AF-2 results from a ligand-induced conformational change involving diverse areas of the LBD (Feng et al. 1998; Selmi-Ruby et al. 1998). Thus, ligand binding serves to switch the receptor from one functional state (e.g. inactive or silencing) to another (e.g. transactivation).

Although much has been learned from studying the structure and function of these receptors, the detailed molecular mechanism(s) of transcriptional regulation by these receptors is not well understood. Efforts to understand the molecular mechanism of transcriptional repression by unliganded TRs and RARs have led to the description (Casanova et al. 1994) and isolation of putative co-repressor proteins SMRT and N-CoR, which interact with the LBD of these receptors in the absence of their ligands (Chen and Evans 1995; Horlein et al. 1995). The recent discovery that both SMRT and N-CoR form complexes with Sin3 and a histone deacetylase suggests that chromatin remodeling by histone deacetylation may play a role in receptor-mediated transcriptional repression (Heinzel et al. 1997; Nagy et al. 1997).

In a somewhat parallel approach, the identification of co-activators has recently received extensive experimental attention in order to elucidate the molecular mechanism(s) of transcriptional activation by nuclear receptors (for a review, see Glass et al. 1997). Identified co-activator proteins primarily belong to two groups: the SRC-1 family and the CBP/p300 family. The SRC-1 family includes SRC-1/NCoA-1 (Onate et al. 1995; Kamei et al. 1996; Torchia et al. 1997), and the related proteins GRIP1/TIF2/NCoA-2 (Hong et al. 1996; Voegel et al. 1996; Hong et al. 1997; Torchia, et al. 1997), and AIB1/p/CIP/ACTR/RAC3/TRAM-1 (Anzick et al. 1997; Chen et al. 1997; Li et al. 1997; Takeshita et al. 1997; Torchia, et al. 1997). The second group of co-activators includes CBP and its homolog p300, which not only influence the activity of nuclear receptors (Chakravarti et al. 1996; Hanstein et al. 1996; Kamei, et al. 1996), but also functionally interact with many transcription factors such as CREB (Chrivia et al. 1993; Kwok et al. 1994; Arany et al. 1995; Lundblad et al. 1995), the Stats (Bhattacharya et al. 1996; Zhang et al. 1996), AP1 (Arias et al. 1994; Bannister and Kouzarides 1995), and p53 (Gu et al. 1997; Lill et al. 1997). There are also co-activator proteins that do not belong to these two groups, such as ARA70 (Yeh and Chang 1996), and PGC-1 (Puigserver et al. 1998). Members of both the SRC-1 family and

CBP/p300 family have been shown to possess histone acetyltransferase (HAT) activities (Bannister and Kouzarides 1996; Ogryzko et al. 1996; Chen, et al. 1997; Spencer et al. 1997), suggesting that chromatin remodeling by histone acetylation is an important mechanism involved in transcriptional activation by ligand-bound nuclear receptors.

Interaction of members of the SRC-1 and CBP/p300 families with nuclear receptors occurs through conserved LxxLL motifs (Heery et al. 1997), which interact with a hydrophobic cleft in the receptor LBD formed as a result of conformational changes mediated by ligand binding (Darimont et al. 1998; Feng, et al. 1998; Nolte et al. 1998). SRC-1/NCoA-1 and GRIP1/TIF2 contain three LxxLL regions or boxes (referred to as LXDs or NR boxes) that differentially interact with nuclear receptors so that different nuclear receptors functionally utilize different LxxLL boxes (Darimont, et al. 1998; McInerney et al. 1998). Thus, ER utilizes the second LxxLL box of SRC-1/NCoA-1 while PR utilizes both the first and second LxxLL boxes for optimal interaction. In contrast, TR and RAR require both the second and third LxxLL boxes for optimal interaction (McInerney, et al. 1998). The specificity of receptor recognition by the different LxxLL boxes of SRC-1/NCoA-1 is primarily mediated by eight amino acid residues C-terminal to the LxxLL motif rather than by the two amino acids (xx) within the motif itself. Thus, while members of the SRC-1 family are capable of interacting with many nuclear receptors, the molecular detail of such interactions differs for each receptor in the number or combination of LxxLL boxes utilized as well as in the critical amino acid residues surrounding the LxxLL motifs.

While much has been learned from the study of known co-activators, a number of key mechanistic questions remain to be answered. For example, many nuclear receptors can recognize common DNA elements (Forman, et al. 1992; Mangelsdorf and Evans 1995; Mangelsdorf, et al. 1995), while not all are capable of regulating genes containing those elements (Schule et al. 1990; Desai-Yajnik and Samuels 1993; MacDonald et al. 1993). Thus, how native target genes containing such elements are selectively regulated by specific receptors is a very important but poorly-understood problem. Although the various LxxLL boxes of SRC-1 and GRIP1 show differential receptor preference (Darimont, et al. 1998; McInerney, et al. 1998), these co-activators

are unlikely to play a primary role in mediating effects that are receptor specific since they appear to interact with all ligand-bound nuclear hormone receptors. Thus, the detailed molecular mechanism(s) underlying receptor-specific regulation of gene expression remains to be elucidated. Whether co-activator(s) might contribute to this specificity is currently unknown.

To further our understanding of the molecular events underlying receptor-activated transcription, we sought to identify additional co-activators using a yeast two-hybrid screening strategy (Gyuris et al. 1993). In this paper, we report the isolation of a novel co-activator for nuclear receptors, designated as NRIF3. Fluorescence microscopy indicates that NRIF3 is a nuclear protein. The yeast two-hybrid and *in vitro* binding assays revealed that NRIF3 interacts specifically with TR and RXR in a ligand-dependent fashion but does not interact with other examined nuclear receptors. Transfection studies indicate that NRIF3 selectively potentiates TR- and RXR-mediated transactivation *in vivo*. NRIF3 encodes a small protein of 177 amino acids and other than an N-terminal LxxLL motif shares no homology with known co-activators. Domain analysis and computer modeling suggest that the receptor specificity of NRIF3 is mediated through a novel C-terminal domain that contains an LxxIL module. These findings provide novel insights into the molecular mechanism(s) of receptor-mediated transcriptional activation as well as the functional specificity of nuclear receptors.

## Results

### *Cloning of the NRIF3 cDNA*

To isolate potential co-activators mediating the transcriptional activation function of nuclear receptors, we employed a yeast two-hybrid screening strategy (Gyuris, et al. 1993). A bait expressing a full length TRα fused to the C-terminus of the LexA DNA binding domain was used to screen a HeLa cell cDNA library cloned into pJG4-5 (Gyuris, et al. 1993). Candidate clones that exhibited a thyroid hormone (T3)-dependent interaction with LexA-TRα were selected and further examined and sequenced. Four novel clones were identified and all were found to exhibit similar interaction with the ligand binding domain (LBD) of TRα as with full length receptor (data

not shown). These clones were designated as NRIF1, 2, 3 and 4 (Nuclear receptor interacting factors). Not surprisingly, the LBD of TRβ was also found to interact with these NRIFs in a T3-dependent manner (data not shown). Among these four isolated NRIFs , NRIF3 was a full length clone. As shown in Figure 1, LexA alone (negative control) does not interact with NRIF3 (as indicated by the low β-galactosidase activity) and incubation with T3 has no effect. Similarly, no interaction was detected between the LexA-TR LBD and B42 alone with or without T3 (data not shown). The LexA-TR LBD also shows little interaction with NRIF3 in the absence of T3. However, incubation with T3 results in strong stimulation of the NRIF3-TR LBD interaction (Fig. 1). The extent of T3-dependent interaction between NRIF3 and LexA-TR LBD was similar to that of Trip1 (Fig. 1), one of the first thyroid hormone receptor interacting factors cloned using a two-hybrid screen (Lee et al. 1995).

*Sequence analysis of NRIF3*

Sequence analysis of the NRIF3 cDNA revealed a single open reading frame (ORF) encoding a polypeptide of 177 amino acids (Fig. 2). NRIF3 shares no homology with members of the SRC-1 and CBP/p300 families. The size of NRIF3 is in sharp contrast to the size of CBP/p300 (around 300 kd), or the SRC-1 family (around 160 kd). NRIF3 contains a putative nuclear localization signal (KRKK), as well as one copy of an LxxLL motif (amino acids 9-13) that was recently identified to be essential for the interaction of a number of putative co-activators with nuclear receptors (Heery, et al. 1997).

A database search identified two highly-related homologs of NRIF3, which were previously designated as β3-endonexin short form and long form (Shattil et al. 1995). The endonexin short form (EnS) was originally isolated from a two-hybrid screen intended to clone factors that interact with the cytoplasmic tail of integrin β3 (Shattil, et al. 1995). The long form (EnL) was then identified as an alternatively spliced product of the same gene. However, the long form does not bind to integrin β3 (Shattil, et al. 1995). Nucleotide sequence comparisons between cDNAs of NRIF3 and endonexin short or long forms indicate that NRIF3 is a third alternatively

spliced product of the same gene (alignment not shown). The precise function(s) of the two endonexin proteins is currently under investigation (S. Shattil, personal communication, also see discussion later).

*NRIF3 localizes to the cell nucleus*

Although a putative nuclear localization signal was found in NRIF3, we considered it important to identify the subcellular location of the NRIF3 protein since extensive homology was found between NRIF3 and the two endonexins. The entire NRIF3 ORF was fused to the C-terminus of green fluorescent protein (GFP) (Cormack et al. 1996). The resulting GFP-NRIF3 fusion protein was expressed in HeLa cells by transient transfection and the subcellular location of the fusion protein was visualized by fluorescence-microscopy. As shown in Figure 3, the control GFP protein is distributed throughout the cell while GFP-NRIF3 is localized exclusively to the nucleus. This result suggests that NRIF3 is a nuclear protein, which is compatible with its putative role as a nuclear receptor co-activator.

*Selective interaction of NRIF3 with liganded nuclear receptors in yeast*

Although NRIF3 was originally cloned using full length TRα as the bait, we later identified that the region of the receptor responsible for NRIF3 binding is its LBD (see Fig. 1). A common feature among most of the known co-activators that show ligand-dependent interaction with nuclear receptors is the presence of the LxxLL motif(s) in their receptor interaction domains. The LxxLL motif appears to be involved in direct contact with a structurally-conserved surface in the ligand-bound LBDs of the receptors (Feng, et al. 1998), which may provide the molecular basis for the broad spectrum of receptor binding by co-activators such as SRC-1 or GRIP1. Since a putative LxxLL motif is also present in NRIF3 (amino acids 9-13), we asked whether NRIF3 also interacts with the LBDs of other nuclear receptors.

The LBDs of several nuclear receptors were examined for interaction with NRIF3 in a yeast two hybrid assay. As shown in Table 1, NRIF3 does not interact with LexA alone (negative

control) with (+) or without (-) ligand. LexA-TR and LexA-RXR show little (if any) interaction with NRIF3 in the absence of their cognate ligands. However, the presence of T3 (for TR) or 9-cis RA (for RXR) results in a strong stimulation of their interaction with NRIF3, as indicated by the induction of β-galactosidase activity (Table 1). Interestingly, when LexA-RAR or LexA-GR was used as the bait, no interaction was detected with NRIF3 in the presence or absence of their cognate ligands (Table 1). The finding that NRIF3 interacts with TR but not RAR was surprising in light of a recent study, which shows that TR and RAR functionally interact with the same LxxLL boxes (boxes 2 and 3) of SRC-1/NCoA-1 (McInerney, et al. 1998). As positive controls, we confirmed that both LexA-RAR and LexA-GR exhibited ligand-dependent interaction with other co-activators that are not receptor-specific (data not shown). Taken together, these results suggest that NRIF3 exhibits differential specificity in its interaction with different nuclear receptors.

*NRIF3 specifically binds to TR and RXR but not to other nuclear receptors in vitro*

To further examine the interaction between NRIF3 and various nuclear receptors as well as to confirm the potential receptor specificity of NRIF3, *in vitro* GST binding assays were performed (Hadzic et al. 1995). $^{35}$S-labeled nuclear receptor, generated by *in vitro* transcription / translation, was incubated with purified GST-NRIF3 or the GST control bound to glutathione-agarose beads. All binding assays were carried out with (+) or without (-) the cognate ligand of the examined receptor. As shown in Figure 4 (top left), TR and NRIF3 interact poorly in the absence of T3. Addition of T3 results in a strong increase in TR binding to GST-NRIF3, confirming that NRIF3 associates with TR in a T3-dependent manner. Using similar binding assays, we also studied the interaction of NRIF3 with six other nuclear receptors. Consistent with our findings from the yeast two-hybrid studies (Table 1), NRIF3 interacts with RXR *in vitro* in a ligand-dependent manner (Fig. 4), but shows little or no binding to other nuclear receptors (RAR, VDR, GR, PR, and ER) in the presence or absence of their cognate ligands (Fig. 4). Taken together, the results of the

yeast two-hybrid (Table 1) and the *in vitro* binding (Fig. 4) assays suggest that NRIF3 possesses a distinct receptor specificity.

*NRIF3 selectively potentiates TR- and RXR-mediated transactivation in vivo*

To examine the potential role of NRIF3 in TR-mediated transactivation, transfection studies were carried out. HeLa cells, which lack endogenous TR (Forman, et al. 1992), were transfected with a vector expressing TR, and a CAT reporter under the control of the ΔMTV basal promoter linked to an idealized inverted repeat (IR) (AGGTCATGACCT) TRE sequence (IR-ΔMTV-CAT) (Forman, et al. 1992), along with either a control plasmid or a vector expressing NRIF3. As shown in Figure 5A, NRIF3 significantly enhances TR-mediated activation of the CAT reporter (typically 2.5- to 3-fold). As a control, we also examined the effect of CBP, a reported co-activator for nuclear receptors (Chakravarti, et al. 1996; Kamei, et al. 1996), and found that its expression results in a similar degree of enhancement as with NRIF3 (around 3-fold) (Fig. 5A).

We also examined another CAT reporter controlled by the Herpes virus thymidine kinase (tk) promoter linked to native rat growth hormone (GH) TRE sequences (Au-Fliegner et al. 1993). NRIF3 was found to also enhance TR-mediated activation of this reporter (about 3.5-fold) (Fig. 5B). In addition, using similar transfection assays, we found that NRIF3 enhances TR-mediated activation of two other reporters, (IR)2-TATA-CAT and DR4-ΔMTV-CAT (data not shown). Therefore, NRIF3 potentiates TR-mediated transactivation in a variety of different TRE / promoter contexts. Taken together, the results of these transfection studies suggest that NRIF3 can function as a co-activator of TR.

To examine whether NRIF3 can also act as a co-activator for RXR, HeLa cells were transfected with the IR-ΔMTV-CAT reporter, whose IR sequence can also function as a strong response element for the RXR(s) and RAR(s) (Forman, et al. 1992; Mangelsdorf and Evans 1995; Qi, et al. 1995). HeLa cells express endogenous RXR(s) and RAR(s), as the activity of the IR-ΔMTV-CAT reporter is strongly stimulated by their cognate ligands, even without co-transfection of any receptor expression plasmid (Fig. 6A, panels 1, 3, and 5). Co-transfection of NRIF3

enhances the activation of this reporter by either 9-cis RA, or LG100153 (Sugawara et al. 1997), an RXR-specific ligand (Fig. 6A, panels 1 and 2; 3 and 4). In contrast, although the RAR-specific ligand TTNPB (Sheikh et al. 1994) also activates the IR-ΔMTV-CAT reporter, co-transfection of NRIF3 has no effect (Fig. 6A, panels 5 and 6). These results indicate that NRIF3 potentiates the activity of endogenous RXR(s) but not RAR(s), which is consistent with the distinct receptor specificity of NRIF3 revealed from the yeast two-hybrid assay (Table 1) and *in vitro* binding studies (Fig. 4).

To further document that NRIF3 can function as a co-activator for RXR, a vector expressing exogenous RXR was co-transfected with IR-ΔMTV-CAT. Exogenous RXR expression enhances the activation of this CAT reporter by either 9-cis RA or LG100153 (comparing Fig. 6B and 6A, panels 1 and 3). This RXR-mediated activation of reporter expression is further stimulated by NRIF3 (Fig. 6B). Finally, we also examined the activation of a DR1-ΔMTV-CAT reporter. This DR1 (AGGTCA n AGGTCA) sequence is thought to be a specific response element for RXR (Kurokawa et al. 1994; Mangelsdorf, et al. 1995). Although we found that this DR1 is a weaker response element than the IR sequence, co-transfection of an RXR expression vector leads to ligand-induced activation of this DR1 reporter, which is also further enhanced by NRIF3 (Fig. 6C).

*NRIF3 does not potentiate the activity of GR, PR, ER, and VDR in vivo*

The selective co-activation of TR and RXR (but not RAR) by NRIF3 is consistent with its distinct binding specificity to these receptors. To further establish that NRIF3 acts as a receptor-specific co-activator, we next examined the effect of NRIF3 on the activity of four additional nuclear receptors, including GR, PR, ER, and VDR, by transfection studies. HeLa cells were transfected with a GRE / PRE-tk-CAT reporter along with a vector expressing either GR or PR. As shown in Figure 7A, cognate hormone treatment results in activation of the CAT reporter. However, expression of NRIF3 has little effect (Fig. 7A). Similar experiments were carried out using ER and ERE-ΔMTV-CAT, or VDR and VDRE-ΔMTV-CAT. As shown in Figure 7B and 7C, NRIF3

was found to have little or no effect on the activity of these receptors as well. Taken together, the combined results of our transfection studies support the notion that NRIF3 is a co-activator with a unique receptor specificity.

*A novel C-terminal domain in NRIF3 is essential for ligand-dependent interactions with TR and RXR*

The LxxLL signature motif has been found to be present in the receptor interacting domain of many identified co-activators such as SRC-1/NCoA-1 and GRIP1/TIF-2 (Heery, et al. 1997). The broad spectrum of receptor binding by co-activators such as SRC-1 suggests that the LxxLL-containing interacting domain may recognize a structurally-similar surface of these LBDs. Indeed, recent structural and functional studies revealed that the LxxLL motif and its nearby flanking amino acids are involved in direct contact with a hydrophobic cleft of the target surface presented by the ligand-bound LBDs of nuclear receptors (Darimont, et al. 1998; Feng, et al. 1998; McInerney, et al. 1998; Nolte, et al. 1998). The fact that NRIF3 also contains an LxxLL motif (amino acids 9-13, see Fig. 2 and Fig. 8A) and exhibits a distinct receptor specificity, raises the possibility that: 1), the motif and surrounding amino acids are involved in mediating the specificity found with NRIF3; or, 2) another region of NRIF3 (alone or in concert with the LxxLL motif region) plays an important role in mediating its receptor specificity.

To explore these questions, we examined whether the endonexin short form (EnS) and endonexin long form (EnL), which contain the same LxxLL motif and flanking amino acids as NRIF3, can interact with nuclear receptors in a yeast two-hybrid assay (Fig. 8). EnS consists of 111 amino acids and is 100% identical to the first 111 residues of NRIF3, while the first 161 amino acids of EnL (170 amino acids) is also 100% identical to the same region in NRIF3 (see Fig. 2 legend and Fig. 8A). Thus, NRIF3 and EnL differ only in their C-terminus, with a unique region of 16 amino acids in NRIF3 or 9 residues in EnL (Fig. 8A). Interestingly, despite their extensive identity with NRIF3, the interaction with liganded TR or RXR is completely abolished in EnS and EnL (Fig. 8B). We also examined other nuclear receptors that do not interact with

NRIF3 and found that they also do not interact with EnS or EnL (data not shown). These results indicate that the unique C-terminal domain in NRIF3 (residues 162-177) is essential for its specific interaction with liganded TR and RXR, while the N-terminal LxxLL motif (amino acids 9-13) and its flanking sequences are not sufficient to allow for detectable receptor interactions.

Although found to be not sufficient for interaction, we examined whether the LxxLL motif of NRIF3 contributes in the NRIF3 / receptor interaction by mutating the first leucine of the LxxLL motif into alanine (L9A) by site-directed mutagenesis. Previous studies have shown that the three leucine residues are essential for an LxxLL module to interact with receptor LBDs, and the replacement of any of them with alanine would abolish the interaction (Heery, et al. 1997). We examined the mutant NRIF3(L9A) for its interaction with TR and RXR in a yeast two-hybrid assay. As shown in Figure 9, the L9A mutant is still capable of ligand-dependent interaction with TR and RXR (~25-fold induction by ligand). However, the introduced mutation reduces the interaction by about 4-fold (for TR) or 14-fold (for RXR). These results suggest that although the LxxLL motif is not absolutely essential for NRIF3 interaction with liganded receptors, it plays a role in allowing an optimum interaction to occur.

*An LxxIL-containing module within the C-terminal domain of NRIF3 appears to specifically interact with ligand-bound TR and RXR but not RAR*

Secondary structure analysis of the C-terminal domain of NRIF3 predicts the formation of an α-helix. Inspection of the putative C-terminal helix revealed an LxxIL motif (amino acids 172-176). A previous study on the second LxxLL box of GRIP1 shows that substitution of the second leucine of the LxxLL core with phenylalanine reduces its affinity for liganded TR by about 50 to 100-fold (Darimont, et al. 1998). In addition, it has been suggested that the second and third (xx) amino acids of the LxxLL core play little if any role in the specificity of interaction with nuclear receptors (McInerney, et al. 1998). To clarify a role for the LxxIL- containing C-terminal helix of NRIF3 in receptor interactions, we modeled the docking of the NRIF3 C-terminal peptide with the ligand-bound LBDs of TRα, RXRα, and RARα. The background information and procedures

used for constructing these models are described in Materials and methods and the results are presented in Figure 10. Our models suggest that the substitution of the second leucine in an LxxLL core with isoleucine still permits a strong hydrophobic interaction with the liganded receptor (Table 2) while a lysine within the LxxIL motif of NRIF3 (K173) and an upstream arginine (R164) contribute to the specificity of NRIF3 binding to TR or RXR through electrostatic interactions. Figure 10 illustrates that the two leucines and one isoleucine (green and cyan) of the LxxIL motif are predicted to be deeply buried into the central cavity of the hydrophobic groove formed by the liganded LBDs of these receptors. In the modeled NRIF3 / RXR and NRIF3 / TR complexes, two specific electrostatic contacts are predicted to further stabilize the interaction: a lysine within the LxxIL motif itself (K173) contacts an acidic residue of the receptor (D295 for RXR or E241 for TR), and an arginine upstream of the LxxIL motif (R164) contacts glutamate(s) of the receptor (E453 for RXR or E399 and E402 for TR). In contrast, no such specific interaction is observed in the modeled NRIF3 / RAR complex.

Based on these models, a prediction of the binding energy between the 17-residue C-terminal peptide of NRIF3 and the LBDs of RXRα, TRα, or RARα was carried out (Table 2), using an improved partitioning binding energy function, with continuum representation of the electrostatics of the system (Schapira et al. 1999). The binding energy was calculated as the sum of a surface, or shape-complementarity term, an electrostatic term, and an entropic term (a constant which accounts for dilution effects and loss of rotational / translational degrees of freedom upon complexation, generally set between +7 and +15 kcal/mol, was omitted since we are more interested in the binding energy difference between the 3 complexes). Table 2 shows that the calculated binding energies for NRIF3 / RXR and NRIF3 / TR are very similar ( ~ -21 kcal/mol). In contrast, the binding energy is more than 20 kcal/mol less favorable for the NRIF3 / RAR complex. This difference comes mainly from the electrostatic term (Table 2). In the NRIF3 / RAR complex, the electrostatic desolvation penalty upon complexation is not counterbalanced by stabilizing specific coulombic interactions, as in the NRIF3 / RXR and NRIF3 / TR complexes. As a result, the electrostatic contribution to the binding energy is much more unfavorable for the

binding of NRIF3 to RAR than to RXR or TR. In summary, the large difference in the total binding energy between NRIF3 / RXR (or TR) and NRIF3 / RAR provides an explanation for the experimentally observed specificity of interaction of NRIF3 with these receptors.

As a control, we carried out a similar modeling procedure using the second LxxLL box within the receptor interacting domain of SRC-1. This LxxLL box has been shown to be required for interaction with TR (McInerney, et al. 1998). A 20-residue helix encompassing this LxxLL box (see Materials and methods) was docked to the co-activator binding site of the TRα LBD, and the binding energy was calculated (Table 2). Interestingly, the surface term of the SRC-1 / TR complex (-42 kcal/mol) was found to be close to the one determined for the NRIF3 / TR complex (-46.5 kcal/mol). This result suggests that the shape complementarity between NRIF3 and TR is comparable to that of SRC-1 / TR. The SRC-1 / TR complex is predicted to be stabilized by a specific interaction between an arginine that is four residues upstream of the LxxLL core, and two glutamates (E399 and E402) of the receptor, which mimics the contact made by R164 of NRIF3 shown in Figure 10. It is interesting to note that a basic residue at this position (i.e. four residues upstream of the LxxLL core) is conserved among the second LxxLL boxes of GRIP1, p/CIP and SRC-1 (Darimont, et al. 1998). Overall, the electrostatic term for SRC-1 / TR (11.3 kcal/mol) is close to the one determined for the NRIF3 / TR complex (12.4 kcal/mol). The total binding energies are also very similar (less than 3 kcal/mol apart, see Table 2).

Altogether, these models and calculations strongly suggest that NRIF3 binds to the co-activator binding site in the LBDs of TR and RXR through its C-terminal helix, where an LxxIL-containing module mimics the LxxLL-containing boxes of SRC-1 and GRIP1. Moreover, the receptor specificity of NRIF3 seems to result from a set of specific electrostatic interactions that involve basic residues both within the LxxIL itself (K173) and from its upstream sequence (R164). The presence (as for TR or RXR) or absence (as for RAR) of such electrostatic interactions likely plays a major role in determining the receptor specificity of NRIF3 that was observed experimentally.

## Discussion

Recent efforts in understanding receptor-mediated transcription have led to the identification of a number of co-activators for nuclear hormone receptors, which can be categorized into two main groups based on overall homology, the SRC-1 family (including SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, AIB1/p/CIP/ACTR/RAC3/TRAM-1) (Onate, et al. 1995; Hong, et al. 1996; Kamei, et al. 1996; Voegel, et al. 1996; Anzick, et al. 1997; Chen, et al. 1997; Hong, et al. 1997; Li, et al. 1997; Takeshita, et al. 1997; Torchia, et al. 1997), and the CBP/p300 family (Chakravarti, et al. 1996; Hanstein, et al. 1996; Kamei, et al. 1996). Other putative co-activators (e.g. ARA70 and PGC-1) that do not belong to the SRC-1 or CBP/p300 families have also been identified (Yeh and Chang 1996; Puigserver, et al. 1998). In addition, p/CAF may also be involved in receptor action through its association with nuclear receptors as well as with other co-activators (Yang et al. 1996; Chen, et al. 1997; Blanco et al. 1998; Korzus et al. 1998). Among these known co-activators, CBP/p300, members of the SRC-1 group, and p/CAF all possess HAT activities (Bannister and Kouzarides 1996; Ogryzko, et al. 1996; Yang, et al. 1996; Chen, et al. 1997; Spencer, et al. 1997).

In this study we report the identification of a novel nuclear protein (NRIF3) which exhibits specific ligand-dependent interactions with TR and RXR but not with RAR, VDR, GR, PR, and ER. Functional studies indicate that NRIF3 potentiates TR- and RXR-mediated transactivation *in vivo* while it exhibits little or no effect on the activity of other examined receptors. Therefore, NRIF3 represents a novel co-activator with a distinct receptor specificity and, thus, may shed light on clarifying the molecular mechanism(s) underlying receptor-specific regulation of gene expression.

A database search indicated that NRIF3 shares no homology with any known co-activators except for a single LxxLL motif. An unusual feature of NRIF3 is its relatively small size, which is in sharp contrast to SRC-1 or CBP/p300. A homology search identified two alternatively spliced isoforms of NRIF3 which were previously designated as β3-endonexin short and long forms (Shattil, et al. 1995). Preliminary studies with these two endonexins indicate that, like NRIF3,

they also localize to the cell nucleus (S. Shattil, personnel communication; Li and Samuels, unpublished data). Interestingly, despite their extensive identities with NRIF3, both the endonexin short and long forms fail to exhibit interaction with liganded nuclear receptors (see Fig. 8). Consistent with this finding, we found that the endonexin short and long forms have little effect on receptor-mediated transcription in transfection studies (data not shown). Therefore, the precise roles of these two endonexins remain to be elucidated. We suggest two not mutually-excluding possibilities. First, since both the endonexin long and short forms appear to localize to the nucleus, it is possible that they may act as co-factors for other transcriptional regulators. Second, since the endonexin short form can interact with the cytoplasmic tail of β3-integrin (Shattil, et al. 1995; Eigenthaler et al. 1997), it may function to communicate signals generated at the plasma membrane to the cell nucleus. An example of a protein which is involved in both cell adhesion and transcriptional regulation is β-catenin (for a review, see Willert and Nusse 1998).

Previous study of the endonexins identified the presence of NRIF3-related mRNAs (by Northern blots) in a wide range of human tissues (Shattil, et al. 1995). Because NRIF3 and the endonexin long form contain almost identical nucleotide sequences and differ only by an alternative splice which results in the removal of a small exon in NRIF3, it is difficult to specifically identify NRIF3 mRNA by Northern blots. A search of the expressed sequence tag database indicates that NRIF3 as well as both the endonexin long and short form mRNAs are expressed. However, the precise determination of cell and tissue distribution of the individual NRIF3 and endonexin short and long forms will require the development of highly selective antibodies. Nevertheless, the wide expression pattern of NRIF3-related mRNAs is consistent with the role of NRIF3 as a co-activator of the TRs, which are also widely expressed (Strait et al. 1990), or the RXRs, which are ubiquitously expressed (Mangelsdorf et al. 1992).

A key question concerning the action of nuclear hormone receptors is to elucidate the molecular events underlying the functional specificity of different receptors in regulating the expression of their target genes. Determinants of specificity include specific ligand binding, selective binding of the receptors to their cognate response elements, as well as specific expression

pattern of different receptors. These determinants alone, however, are not always sufficient to explain the extent of specificity observed for members of the nuclear receptor family. For example, several members of the thyroid hormone / retinoid receptor subfamily may bind similarly to common DNA elements while target genes containing those elements are only selectively activated by certain receptors (Desai-Yajnik and Samuels 1993; MacDonald, et al. 1993). Therefore, it is likely that additional factors (determined by cell / promoter contexts) are involved in determining receptor functional specificity. In this respect, most known co-activators do not appear to be receptor-specific. For example, members of the SRC-1 family and CBP/p300 interact with and appear to be involved in the action of many nuclear receptors (Chakravarti, et al. 1996; Kamei, et al. 1996; Chen, et al. 1997; Hong, et al. 1997). Two known co-activators that may be involved in receptor-specific functions are ARA70 and PGC-1. The androgen receptor co-activator ARA70 has been reported to potentiate the activity of AR more efficiently than for other nuclear receptors (Yeh and Chang 1996). However, whether ARA70 can associate with other receptors remains to be thoroughly examined. The expression of PGC-1 is mainly restricted to the brown fat tissue and is thought to be directly involved in the regulation of thermogenesis by PPARγ (Puigserver, et al. 1998). Nevertheless, PGC-1 exhibits a relatively broad spectrum of binding to different nuclear receptors. Therefore, the identification of NRIF3 represents the first example of a co-activator with such a clearly-defined receptor specificity.

The receptor-specificity of NRIF3 raises an interesting question about its molecular mechanism. Domain analysis suggests that the LxxLL motif (amino acids 9-13) and its flanking sequences in NRIF3 are not sufficient for interaction with liganded nuclear receptors. In fact, such interaction is completely abolished in the endonexin long form, an alternatively spliced product which has the same LxxLL motif and contains the first 161 amino acids (out of a total of 177 amino acids) of NRIF3. This result suggests that a putative domain consisting of the last 16 amino acids of NRIF3 (residues 162-177) is essential for its interaction with liganded receptors. Inspection of this C-terminal region of NRIF3 indicates that it contains an LxxIL motif (amino acids 172-176) and secondary structure analysis predicts the formation of an α-helix. The

predicted helix structure and the similarity of LxxIL to the canonical LxxLL raise the possibility that this LxxIL-containing region may play a direct role in the specific interaction of NRIF3 with ligand-bound TR or RXR.

Modeling of the docking of a C-terminal peptide of NRIF3 containing the LxxIL motif to various ligand-bound LBDs (Fig. 10) and calculation of the resulting binding energies (Table 2) reveal several important points. First, the same hydrophobic groove in the ligand-bound LBD, which has been shown by previous studies to be the binding site for co-activators such as SRC-1/NCoA-1 or GRIP1 (Darimont, et al. 1998; Feng, et al. 1998; Nolte, et al. 1998), appears also to be a suitable site for the docking of the C-terminal helix of NRIF3. Thus, the utilization of the complementary pair of an α-helix (in the co-activator) and a hydrophobic groove (in the receptor) for interaction seems to be a general scheme that also applies to NRIF3. Second, the binding energy estimated for the NRIF3 peptide and the TR LBD (or RXR LBD) is similar to the one calculated for the second LxxLL box of SRC-1/NCoA-1 and the TR LBD. Third, the receptor specificity of the NRIF3 peptide appears to be determined by electrostatic interactions formed by K173 and R164 of the NRIF3 peptide with specific acidic residues of the ligand-bound LBDs of TR and RXR. Such charge interactions do not appear to occur in the similarly modeled ligand-bound LBD of RAR. This results in a predicted ~20 kcal/mol difference in the binding energy between NRIF3 / TR (or RXR) and NRIF3 / RAR, which would explain the differential interaction patterns of NRIF3 with these receptors. In summary, the C-terminal region of NRIF3 is predicted to directly contact the hydrophobic groove of liganded TR and RXR with high affinity, where specific basic amino acids upstream of and contained within the LxxIL motif play an important role in determining the binding affinity and / or specificity. While the models presented in Figure 10 need to be tested by more systematic mutagenesis studies in the future, we have examined two TR AF-2 mutants (Selmi-Ruby, et al. 1998) and found that in both cases, ligand-dependent interaction with NRIF3 was abolished (Li and Samuels, unpublished data). This result supports the notion that the integrity of the hydrophobic groove (in which the AF-2 helix of the receptor is an important constituent) is essential for NRIF3 binding, which is predicted by our models.

Although the N-terminal LxxLL motif (amino acids 9-13) is insufficient alone to mediate an interaction with TR or RXR (see Fig. 8), it can influence the interaction of NRIF3 with these receptors, as the NRIF3 L9A mutant retains significant but nevertheless reduced association with liganded TR or RXR (Fig. 9). Thus, NRIF3 appears to employ at least two regions in interacting with liganded TR or RXR, with the C-terminal helix playing an essential role and the N-terminal LxxLL motif playing a secondary role. A simplified explanation for these findings would be that the C-terminal helix of NRIF3 provides a major surface for receptor binding, and is responsible for most if not all of the receptor specificity of NRIF3, while the N-terminal LxxLL motif makes some minor contact with either the same receptor molecule, or more likely, with the other partner of a homodimer or heterodimer to further stabilize the NRIF3-receptor interaction. An example of a co-activator molecule employing two separate regions to interact with the two partners of a receptor dimer has been documented in the recently solved crystal structure of liganded PPARγ complexed with SRC-1/NCoA-1 (Nolte, et al. 1998). Without the C-terminal domain of NRIF3, the affinity of the N-terminal LxxLL motif alone with liganded TR or RXR is insufficient for receptor interaction. In summary, our results suggest that novel surfaces are involved in NRIF3 / receptor interaction and in determining receptor specificity of NRIF3. It is tempting to speculate that the employment of a core motif that is a variant of the canonical LxxLL (such as LxxIL) and the utilization of specific residues contained within and surrounding the core motif in determining receptor specificity could be a strategy that not only applies to NRIF3, but also to other yet to be defined receptor interacting factors as well.

Accumulating evidence suggests that the actions of transcriptional activating proteins are (usually) mediated by multi-protein complexes (for a review, see Orphanides et al. 1996) and such a scheme is also likely for nuclear receptors. For example, biochemical evidence suggests that multi-protein complexes associate with liganded TR and VDR (Fondell et al. 1996; Rachez et al. 1998; Yuan et al. 1998). Interestingly, many of the proteins identified in these studies are not known co-activators. While the study of known co-activators such as CBP/p300 and the SRC-1 family has suggested that histone acetylation may play an important role in receptor-mediated

transactivation (Bannister and Kouzarides 1996; Ogryzko, et al. 1996; Chen, et al. 1997; Spencer, et al. 1997), detailed elucidation of the transactivation mechanism(s) by these receptors awaits the identification and study of additional co-factors involved in the transactivation process.

Our results with NRIF3 suggest that transcriptional activation by nuclear receptors may employ receptor-specific co-activator(s) in addition to the generally-utilized co-activators such as CBP or SRC-1. Therefore, co-activators with NRIF3-like properties may contribute to the functional specificity of nuclear receptors *in vivo*. Based on our results with NRIF3 and previous studies of nuclear receptor action, we suggest a "combinatorial specificity model" where a co-activation complex is likely composed of two kinds of factors: "general factors" that interact with and are involved in the action of many nuclear receptors (such as CBP or SRC-1), and "specific factors" that exhibit receptor specificity (such as NRIF3). In addition to their interaction with the liganded receptor, co-activators may also communicate with each other within the co-activation complex through protein-protein interactions (e.g. CBP/p300 can interact with SRC-1/NCoA-1 or p/CIP) (Kamei, et al. 1996; Yao et al. 1996; Torchia, et al. 1997). The combinatorial actions of "specific factors" and other partners involved in the transactivation process may facilitate the recruitment of specific co-activation complexes for different receptors (under different cell / promoter / HRE contexts), which would provide an important mechanistic layer for receptor functional specificity. An advantage of employing such a combinatorial strategy is that a broad array of diversity can be generated from a relatively small number of involved factors. Further study of NRIF3 with known and possibly other yet to be identified co-activators, as well as analysis of the interplay among these co-activators should provide important insights into the molecular mechanism(s) underlying the specificity of receptor-mediated regulation of target gene expression.

## Materials and methods

*Isolation of NRIFs and the yeast two-hybrid assay*

The Brent two-hybrid system (Gyuris, et al. 1993) was employed to isolate candidate cDNA clones interacting with LexA-TRα in a ligand-dependent fashion. Full length chicken TRα (cTRα) was fused in frame to the C-terminus of the LexA DBD in pEG202 (Gyuris, et al. 1993). The LexA-TRα bait, the LacZ reporter (pSH18-34), and a pJG4-5 based HeLa cell cDNA library were transformed into the yeast strain EGY48 (Gyuris, et al. 1993). The transformants were selected on Gal/Raf/X-gal medium in the absence of leucine and were further screened for the expression of LacZ in the presence of 1μM T3. Blue colonies were picked and re-examined for T3-dependent expression of LacZ. Positive yeast clones were then selected and plasmids harboring candidate prey cDNAs were isolated. Individual candidate prey plasmid was then amplified in E. coli and re-transformed into the original yeast strain to confirm the interaction phenotype. The cDNA inserts were then sequenced using an automatic sequencer. Four novel clones (NRIF1, 2, 3, and 4) were obtained. Among them, NRIF3 was a full length clone.

Wild type NRIF3, the endonexin long form (EnL) and short form (EnS), and the L9A NRIF3 mutant were examined for their interaction with various nuclear receptors in a yeast two-hybrid assay. The following receptor baits were used: LexA-cTRα LBD, LexA-hTRβ LBD, LexA-hRARα LBD, LexA-hRXRα LBD, and LexA-hGR LBD. Yeast cells harboring appropriate plasmids were grown in selective media with Gal/Raf in the presence or absence of cognate ligand (1 μM T3 for TR, all trans or 9-cis RA for RAR, 9-cis RA for RXR, and 10 μM deoxycorticosterone for GR) overnight before β-galactosidase activity was assayed using o-Nitrophenyl β-D-Galactopyranoside as the substrate. β-galactosidase units are expressed as (O.D. 420 nm x 1000) / (minutes of incubation x O.D. 600 nm of yeast suspension).

*Fluorescence Microscopy*

Full length NRIF3 was cloned into the GFP fusion protein expression vector pEGFP (Clontech). The resulting GFP-NRIF3 vector and the control plasmid pEGFP were transfected into HeLa cells

by calcium phosphate co-precipitation. Cells were incubated at 37°C for 24 hours before the examination with a fluorescence microscope to determine the subcellular location of GFP-NRIF3 or the GFP control.

*In vitro binding assay*

Full length NRIF3 was cloned into pGEX2T, a bacterial GST-fusion protein expression vector (Pharmacia). The GST-NRIF3 fusion protein was expressed in E. coli and affinity purified using glutathione-agarose beads (Hadzic, et al. 1995). $^{35}$S-labeled full length cTRα, hRARα, hRXRα hVDR, hGR, hPR, and hER were generated by *in vitro* transcription / translation using a reticulocyte lysate system (Promega). Binding was performed as previously described (Hadzic, et al. 1995), using the following buffer: 20 mM Hepes (pH 7.9), 1 mM $MgCl_2$, 1 mM DTT, 10% Glycerol, 0.05% Triton X-100, 1 μM $ZnCl_2$, and 150 mM KCl. Appropriate ligands were added into the binding reaction when indicated: 1 μM T3 for TR, 1 μM all trans RA or 9-cis RA for RAR, 1 μM 9-cis RA for RXR, and 150 nM 1,25-$(OH)_2$ VitD3, dexamethathone, progesterone, or estradiol for VDR, GR, PR, or ER. After the binding reaction, the beads were washed three times and the labeled receptors bound to the beads were examined in 10% SDS-PAGE followed by autoradiography. Five percent of the $^{35}$S-labeled receptor input was also electrophoresed in the same gel.

*Transfection studies*

Most reporters used in this study have been described previously, including IR-ΔMTV-CAT, DR4-ΔMTV-CAT, GH-TRE-tk-CAT, IR+3 (ERE)-ΔMTV-CAT (Umesono, et al. 1991; Forman, et al. 1992; Au-Fliegner, et al. 1993). A DR1-ΔMTV-CAT reporter responsive to RXR was obtained from Ron Evans. A GRE / PRE-tk-CAT reporter was obtained from Gunther Schutz. The (IR)2-TATA-CAT was constructed in our laboratory by cloning two copies of the IR sequence (AGGTCA TGACCT) upstream of a TATA element derived from the tk promoter. A hVDR expression vector and the VDRE-ΔMTV-CAT containing the VDRE from the osteocalcin promoter

were obtained from J. Wesley Pike. Vectors expressing cTRα, hRARα, hRXRα, rGR, hPR, and hER have been described previously (Conneely et al. 1986; Miesfeld et al. 1986; Giguere et al. 1987; Waterman et al. 1988; Mangelsdorf et al. 1990; Forman, et al. 1992). The NRIF3 expression vector was constructed by cloning full length NRIF3 into a pExpress vector (Forman, et al. 1992). Appropriate plasmids were transfected into HeLa cells by calcium phosphate co-precipitation using 25-100 ng of the receptors, 250-500 ng of the CAT reporters, and 750 ng of the NRIF3 or control pExpress vector. After transfection, cells were incubated at 37°C (with or without cognate ligands) for 42 hours before being harvested. CAT assays were carried out as previously described (Hadzic, et al. 1995). Relative CAT activity was determined as the percent acetylation of substrate per 30 μg of cell protein in a 15 hour incubation at 37°C. The results were calculated from duplicate or quadruplicate samples and the variation among samples was less than 10%.

*Domain and mutagenesis analyses*

To construct pJG4-5 derived vectors expressing EnL or EnS, the pJG4-5/NRIF3 plasmid was digested with NcoI and XhoI, and the resulting vector fragment was gel-purified. This fragment was then ligated to an EnL or EnS insert generated from pExpress-EnL or pExpress-EnS by an NcoI / SalI double digest. The resulting pJG4-5/EnL or EnS plasmids were confirmed by sequence analysis. The L9A mutant form of NRIF3 was generated by site-directed mutagenesis using a PCR-based method, and the mutation was confirmed by sequence analysis. pJG4-5 derived vectors expressing EnL, EnS, or the L9A NRIF3 mutant form were transformed into yeast strains harboring the LacZ reporter (pSH18-34) and appropriate bait plasmids (LexA-TR, LexA-RAR, LexA-RXR, and LexA-GR). Transformants were subjected to quantitative assays of β-galactosidase activity as described earlier.

*Docking of co-activator peptides to receptors*

We built a model of the interaction between the 17-residue C-terminal peptide of NRIF3 (KASRHLDSYEFLKAILN) and the LBDs of RXRα, TRα or RARα. An LxxIL motif within the NRIF3 peptide is underlined. A similar modeling procedure was carried out on a 20-residue peptide (SLTERHKILHRLLQEGSPSD) of the second LxxLL box of SRC-1 (McInerney, et al. 1998). The crystal structures of ligand-bound TRα (Wagner et al. 1995) and RARγ (Renaud et al. 1995). were used, while the conformation of liganded RXRα was modeled from its inactive state (see below). We hypothesized that the LxxIL motif of the C-terminus of NRIF3 contacts the co-activator binding site of the nuclear receptors, and the automatic docking procedure was carried out towards this site (Totrov and Abagyan 1994; Strynadka et al. 1996; Totrov and Abagyan 1997). Two critical features of the interaction between the LBDs of nuclear hormone receptors and their co-activators were used to build the models: 1) The "charge clamp", initially observed in the complex between SRC-1 and PPARγ (Nolte, et al. 1998), where a conserved glutamate and lysine at opposite ends of the hydrophobic cavity of the receptors contact the backbone of the co-activator's LxxLL box. This feature enabled the orientation of the NRIF3 helical peptide and, 2) The finding that the leucines of the LxxLL motif of SRC-1 are buried into the hydrophobic cavity of the receptor. This feature makes predictions of the side of the NRIF3 peptide which faces the receptor.

The co-activator peptides were assigned a helical secondary structure, the backbone $\phi$ and $\psi$ angles being -62 and -41 degrees, respectively. The $\omega$ angle was set to 180 degrees. Loose distance restraints were set between the "charge clamp" of the receptors (Nolte, et al. 1998) and $C^{\alpha}$ atoms of the peptide. The energy of the complex was minimized in the internal coordinate space using the modified ECEPP/3 potentials. The subset of the variables minimized with the ICM method (Abagyan et al. 1994; Strynadka, et al. 1996; Totrov and Abagyan 1997), included the side-chains of the receptor, six positional variables of the helix and the side-chain torsion angles of the helix.

*Modeling of the conformation of ligand-bound RXRα and RARα*

9-cis RA was docked into the crystal structure of the binding pocket of RARγ with the flexible docking procedure. First, the continuously flexible ligand was docked into a set of five grid potentials (small probe van der Waals grid, large probe van der Waals grid, electrostatic potential, hydrogen bonding potential and the hydrophobic potential). Second, the ligand was refined together with the flexible receptor side-chains (Totrov and Abagyan 1997). The model of the RXRα / 9-cis RA complex was derived from the RARγ / 9-cis RA complex by replacement of the side-chains, followed by the energy optimization.

In order to analyze the co-activator helix binding of RARα, we built a model of RARα LBD based on the close homology to the known RARγ isoform. The two isoforms share 80% sequence identify. The four residues non-identical between the two isoforms, which are in the vicinity of the co-activator binding pocket were changed to the RARα isoform type (R247Q, P407S, R413Q, P419S), and the energy was minimized. Peptide docking results were found to be similar for RARα and RARγ

*Binding energy calculation:*

The binding energy was calculated by the partitioning method as described elsewhere (Schapira, et al. 1999). Briefly, the binding energy function is partitioned into three terms: the surface (or hydrophobic) term, determined as the product of the solvent accessible surface by a surface tension of 30 cal/mol/Å$^2$, the electrostatic term, calculated by a boundary element algorithm, with a dielectric constant of 8, and the entropic term, which results from the decrease in conformational freedom of residue side-chains partially or completely buried upon complexation.

## Acknowledgments

We are grateful to Richard Goodman, Bert O'Malley, Ming-Jer Tsai, Michael Garabedian, J. Wesley Pike, Gunther Schutz, Ron Evans, and David Moore for plasmids and Richard Heyman of Ligand, Inc. for providing the retinoids. We thank Sanford Shattil for GFP-EnL and GFP-EnS, Gordon Fishell for help with fluorescence microscopy, Chun Wong, Ula Huang, Sidney Guo, and Paul Modlinger for experimental assistance, and Bruce Raaka and Fred Stanley for advice with graphic preparations. This research was supported by NIH grant DK16636 (to H.H.S.), NRSA postdoctoral fellowship award DK09581 (to D.S.L.), DOD grant DAMD179818133, NIH grant GM5541801 and DOE grant DEFG0296ER62268 (to R.A. and M.S.), and NIH Short Term Training Grant for Students in Health Professional Schools DK07421 (E.L.). V.D-Y. was supported in part by The Aaron Diamond Foundation (HRI817-5332F). H.H.S., V.D-Y., and R.A. are members of the NYUMC Cancer Center (CA16087). Sequence analysis and database searches were through the NYUMC Research Computing Resource which received support from the National Science Foundation (DIR-8908095).

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**Table 1.** *Interaction of NRIF3 with nuclear receptors in yeast*

| Bait | Prey | β-galactosidase activity | | |
|---|---|---|---|---|
| | | Ligand | | Fold Stimulation |
| | | – | + | |
| LexA | NRIF3-B42 | 2.3 | 1.9 | 0.8 |
| LexA-TR | NRIF3-B42 | 1.8 | 125 | 69 |
| LexA-RAR | NRIF3-B42 | 0.1 | 0.1 | 1 |
| LexA-RXR | NRIF3-B42 | 0.2 | 63 | 315 |
| LexA-GR | NRIF3-B42 | 0.8 | 0.6 | 0.8 |

The LacZ reporter activity was determined for yeast strains (EGY48) harboring the indicated bait and prey plasmids in the presence (+) or absence (-) of cognate ligands as described in Materials and methods. See text for detailed explanations.

**Table 2.** *Binding energy prediction for the interaction of the 17-residue C-terminal peptide of NRIF3 with ligand-bound LBDs of RXR, TR, and RAR*

| | Hydrophobic | Electrostatic | Entropic | Total |
|---|---|---|---|---|
| NRIF3 / RXR | -39.3 | 6.7 | 11.4 | -21.2 |
| NRIF3 / TR | -46.5 | 12.4 | 13.2 | -20.9 |
| NRIF3 / RAR | -39.9 | 30.7 | 11.1 | 1.8 |
| SRC-1 / TR | -42.0 | 11.3 | 12.6 | -18.0 |

For each of the modeled peptide / receptor complexes, contributions by the surface (hydrophobic) term, the electrostatic term, and the entropic term were calculated as described in Materials and methods. The total binding energy was then calculated as the sum of these three terms. SRC-1 / TR was used as a control. The energy unit used in the table is kcal/mol. See text for details.

## Figure legends

**Figure 1.** Hormone-dependent interaction of NRIF3 with the ligand binding domain (LBD) of TR. Induction of β-galactosidase activity by thyroid hormone (T3) was measured in the yeast strain EGY48 transformed with a bait vector expressing LexA-cTRα LBD and the prey plasmid expressing NRIF3 fused to the B42 activation domain (Gyuris, et al. 1993). The bait LexA alone was used as the negative control. The prey B42-Trip1 was used as the positive control.

**Figure 2.** Nucleotide and deduced amino acid sequences of NRIF3. Only part of the cDNA sequence is shown. A putative nuclear localization signal (KRKK) is underlined. The putative LxxLL motif is shown with a double underline. NRIF3 and the β3-endonexin long form (EnL) share 95% identity. They differ only in the C-terminus where the last 16 amino acids (dot underlined) in NRIF3 is replaced with 9 different amino acids (GQPQMSQPL) in the β3-endonexin long form. The short form of β3-endonexin consists of 111 amino acids and is 100% identical to the first 111 amino acids of NRIF3 or the β3-endonexin long form.

**Figure 3.** NRIF3 is a nuclear protein. HeLa cells were transfected with a expression vector for GFP (left panel) or GFP-NRIF3 (right panel). The cellular location of the expressed proteins was visualized by fluorescence microscopy.

**Figure 4.** Characterization of the NRIF3 interaction with nuclear receptors *in vitro*. $^{35}$S-labeled full length receptor (cTRα, hRARα, hRXRα, hVDR, hPR, hGR, or hER) was incubated with affinity purified GST control or GST-NRIF3 linked to glutathione-agarose beads. The binding was performed in the absence (-) or presence (+) of cognate ligands as described in Materials and methods. After incubation and washing, the bound receptors were analyzed in 10% SDS-PAGE and detected by autoradiography. The input lane in each binding assay represents 5% of the total

$^{35}$S-labeled receptor used in each incubation. GST-RXR was used as a positive control for RAR binding.

**Figure 5.** NRIF3 enhances TR-mediated transactivation *in vivo*. HeLa cells were transfected with a vector expressing cTRα, and the IR-ΔMTV-CAT reporter (A) or the GH-TRE-tk-CAT reporter (B) in the presence (filled columns) or the absence (hatched columns) of 1 μM T3. The vector expressing NRIF3 or the empty control vector were co-transfected to examine the effect of NRIF3 on TR-mediated activation. In (A), the effect of CBP was compared to that of NRIF3.

**Figure 6.** NRIF3 functions as a co-activator for RXR but not RAR. (A) NRIF3 potentiates the activity of endogenous RXR(s) but not RAR(s). HeLa cells were transfected with the IR-ΔMTV-CAT reporter (without any receptor expression vector) to examine the activation by endogenous retinoid receptors. The NRIF3 expression vector or the empty control vector were co-transfected to examine the effect of NRIF3 on the activity of endogenous RXR(s) or RAR(s). Relative CAT activity was determined in the presence (filled columns) or absence (hatched columns) of indicated ligands (1 μM). (B), (C) NRIF3 potentiates the activity of exogenously expressed RXR. A vector expressing hRXRα was co-transfected into HeLa cells with the IR-ΔMTV-CAT reporter (B) or the DR1-ΔMTV-CAT reporter (C), in the presence (filled columns) or absence (hatched columns) of indicated ligands (1 μM). The effect of NRIF3 on RXR-mediated transactivation was similarly examined as in (A).

**Figure 7.** NRIF3 does not potentiate the activity of GR, PR, ER, or VDR. HeLa cells were transfected with the following CAT reporters and appropriate receptor expression vectors: GRE / PRE-tk-CAT and rGR or hPR (A), ERE-ΔMTV-CAT and hER (B), VDRE-ΔMTV-CAT and hVDR (C). Cells were incubated in the presence (filled columns) or absence (hatched columns) of 100 nM dexamethathone for GR, progesterone for PR, estradiol for ER, and 1,25-$(OH)_2$-VitD3

for VDR. Co-transfection of NRIF3 was found to have little effect on the activity of these receptors.

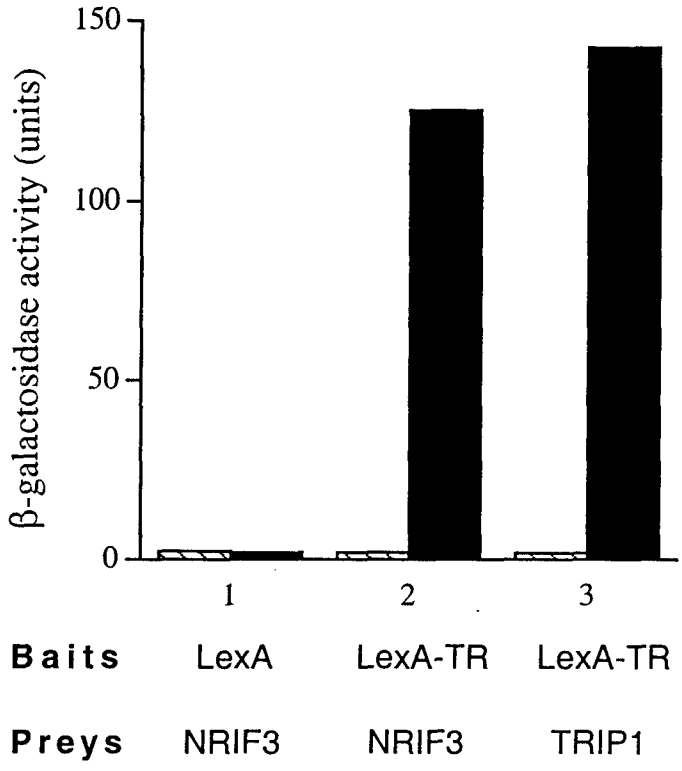
**Figure 8.** The C-terminal domain of NRIF3 is essential for the interaction with liganded TR or RXR. (A) schematic comparison of NRIF3 with EnS and EnL. EnS is 100% identical to the first 111 amino acids of NRIF3 or EnL (open box). The region from amino acid 112 to 161 is 100% identical between NRIF3 and EnL (dotted box). NRIF3 and EnL differ in their C-terminus (16 amino acids in NRIF3, hatched box; and 9 amino acids in EnL, filled box). The positions of the LxxLL motif and a putative nuclear localization signal (KRKK) are also indicated. (B) NRIF3 (N), EnS (S), or EnL (L) was examined for interaction with LexA-TR or LexA-RXR in a yeast two-hybrid assay as described in Materials and methods. The assays were performed in the absence (hatched columns) or the presence (filled columns) of 1 μM T3 (for TR) or 9-cis RA (for RXR).

**Figure 9.** The LxxLL motif of NRIF3 is required for optimum interaction with TR and RXR. Wild type NRIF3 (WT) or the L9A NRIF3 mutant (L9A) was examined for interaction with LexA-TR or LexA-RXR in a yeast two-hybrid assay as described in Materials and methods. β-galactosidase activities were determined in the absence (filled columns) or presence (dotted columns) of cognate ligands (1 μM T3 for TR, 1 μM 9-cis RA for RXR).

**Figure 10.** A structural model for the receptor specificity of NRIF3. The docking of the C-terminal helix of NRIF3 which contains an LxxIL module to the ligand-bound LBDs of RXRα, TRα, and RARα was carried out as described in Materials and methods. The side chains of the two leucines (green) and one isoleucine (cyan) of the LxxIL core fit within a hydrophobic groove on the surface of the liganded LBDs. Positively charged side chains contributed by a lysine within the LxxIL core (K173) and an arginine upstream of the core (R164) are shown in blue. Areas of negative electrostatic potential on the surfaces of the LBDs are shown in red. Specific acidic

residues of the RXR and TR LBDs that contact K173 and R164 of the NRIF3 helix are indicated by red arrows. It was calculated that such electrostatic interactions (which are absent in the NRIF3 / RAR model) play a major role in determining the specificity of NRIF3 (see text and Table 2 for details).

FIGURE 1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LexA-TR
Preys
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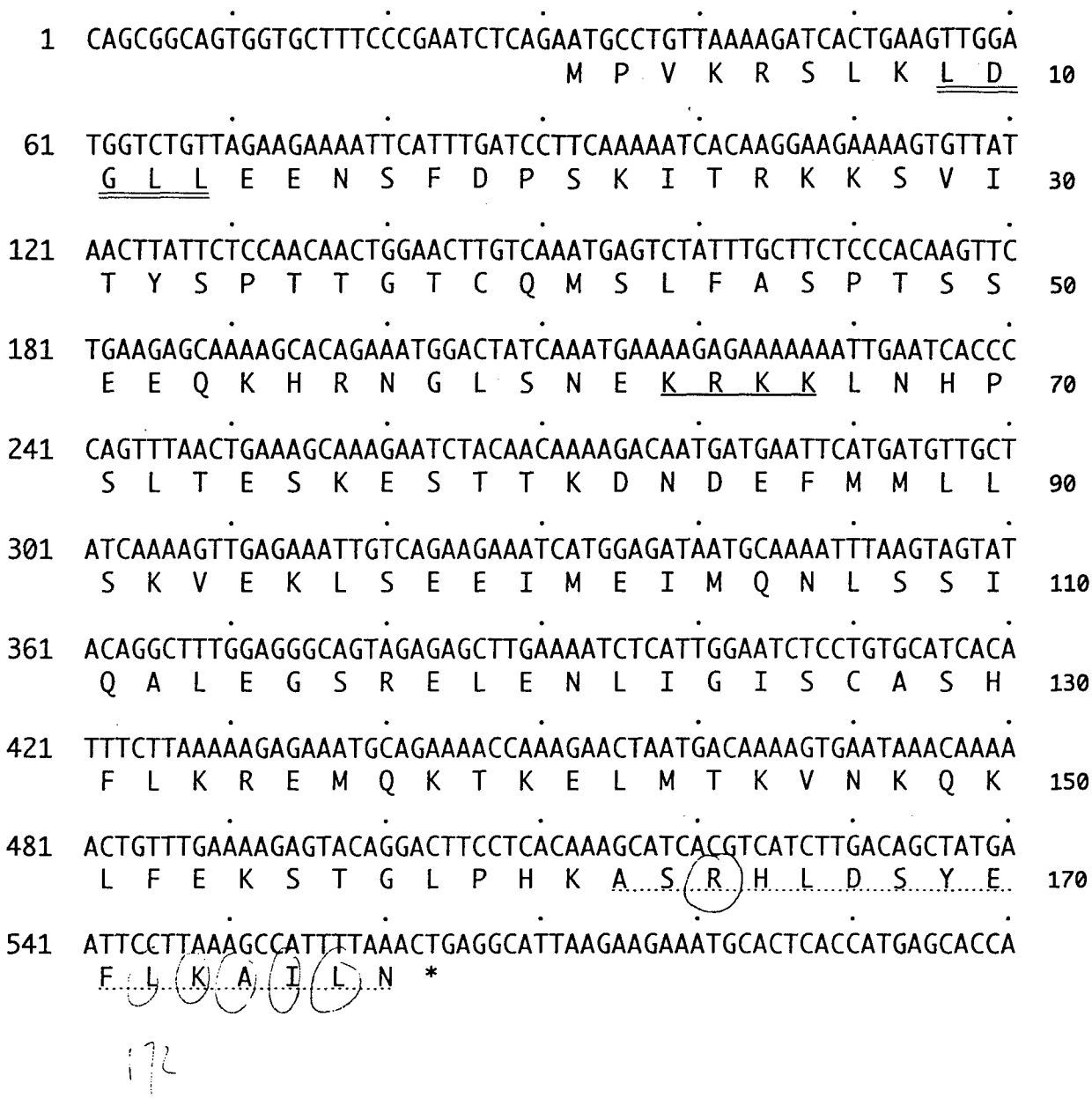
NRIF3
TRIP1

FIGURE 2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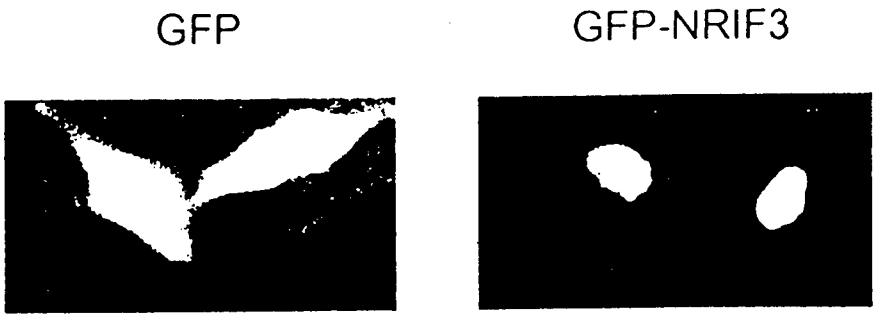
F L K A I L N *

172

FIGURE 3



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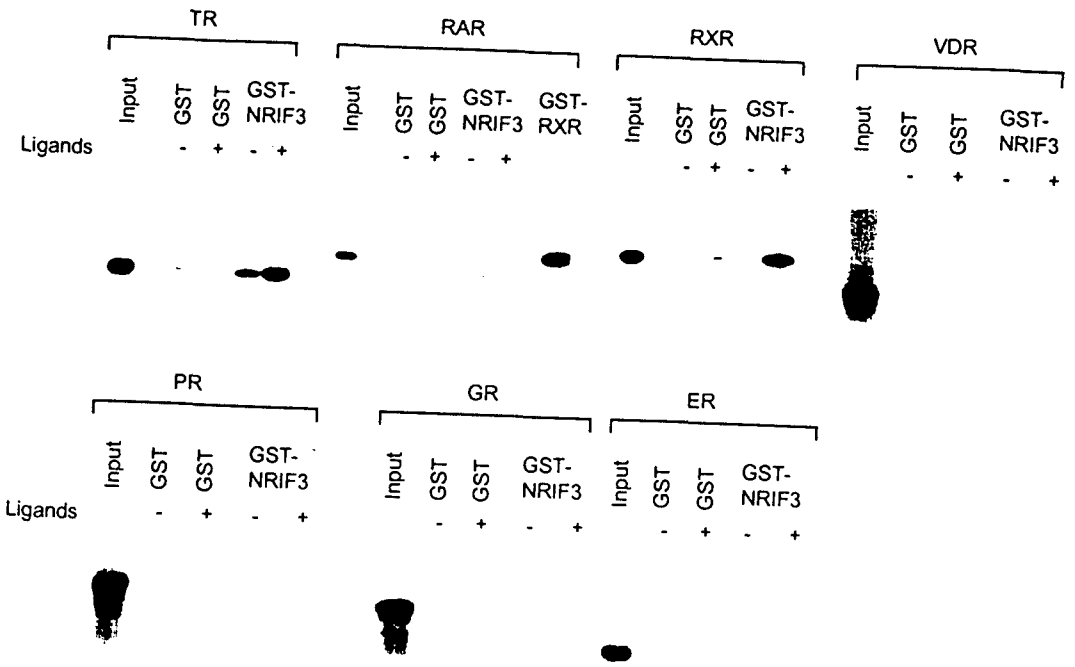

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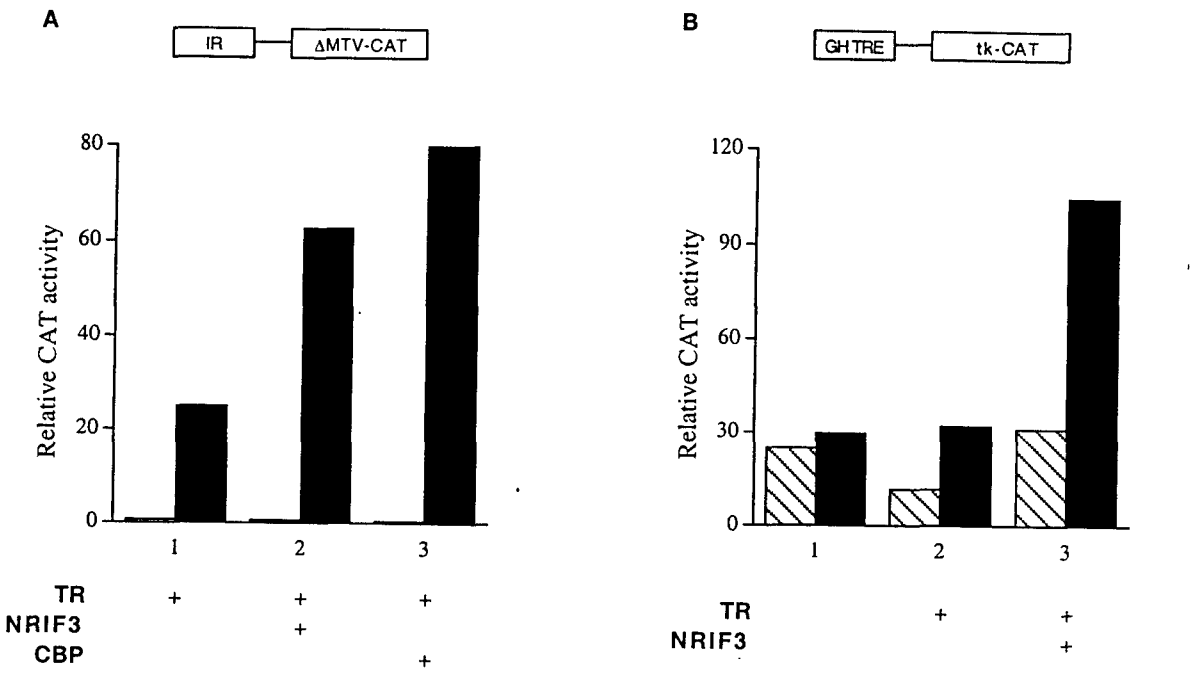
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40
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2
3
TR
NRIF3
CBP
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GHTRE
tk-CAT
Relative CAT activity
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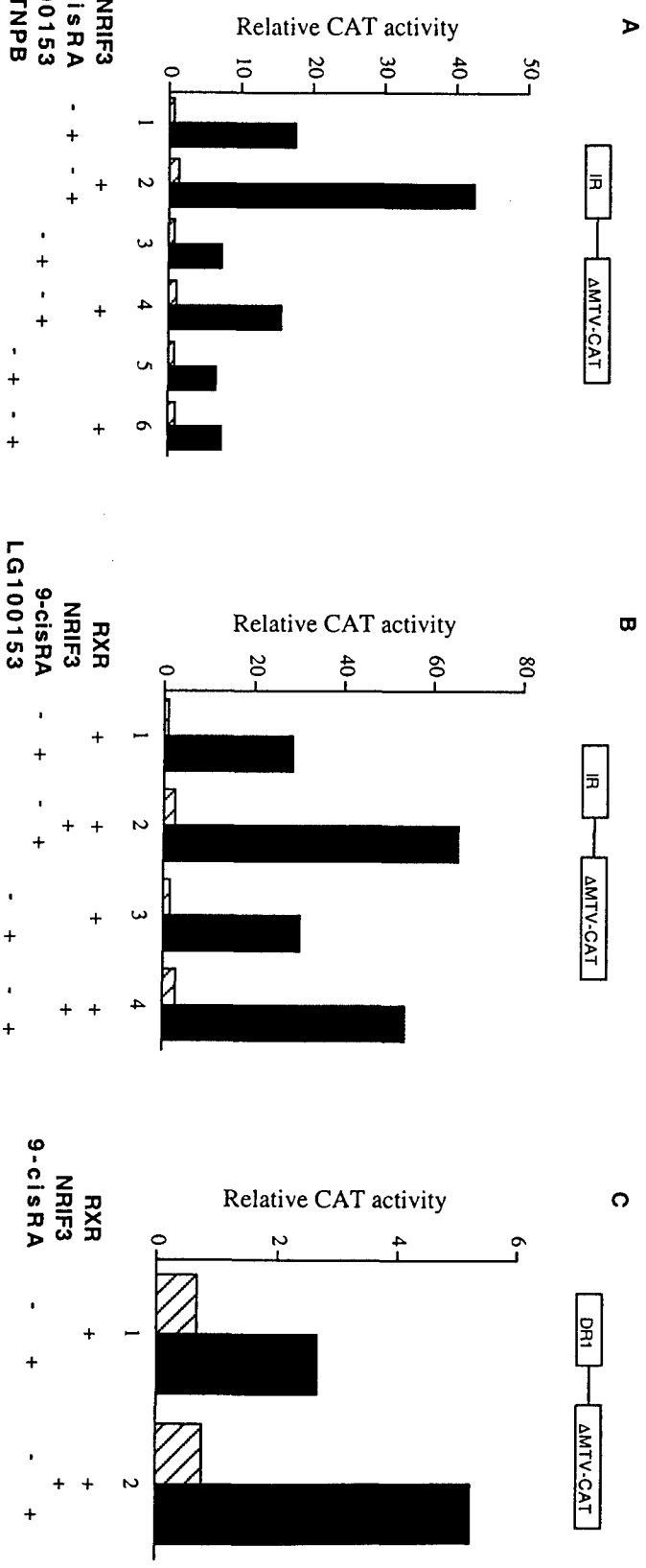
90
60
30
0
1
2
3
TR
NRIF3

FIGURE 6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2
3
4
5
6
B
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40
60
80
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NRIF3
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1
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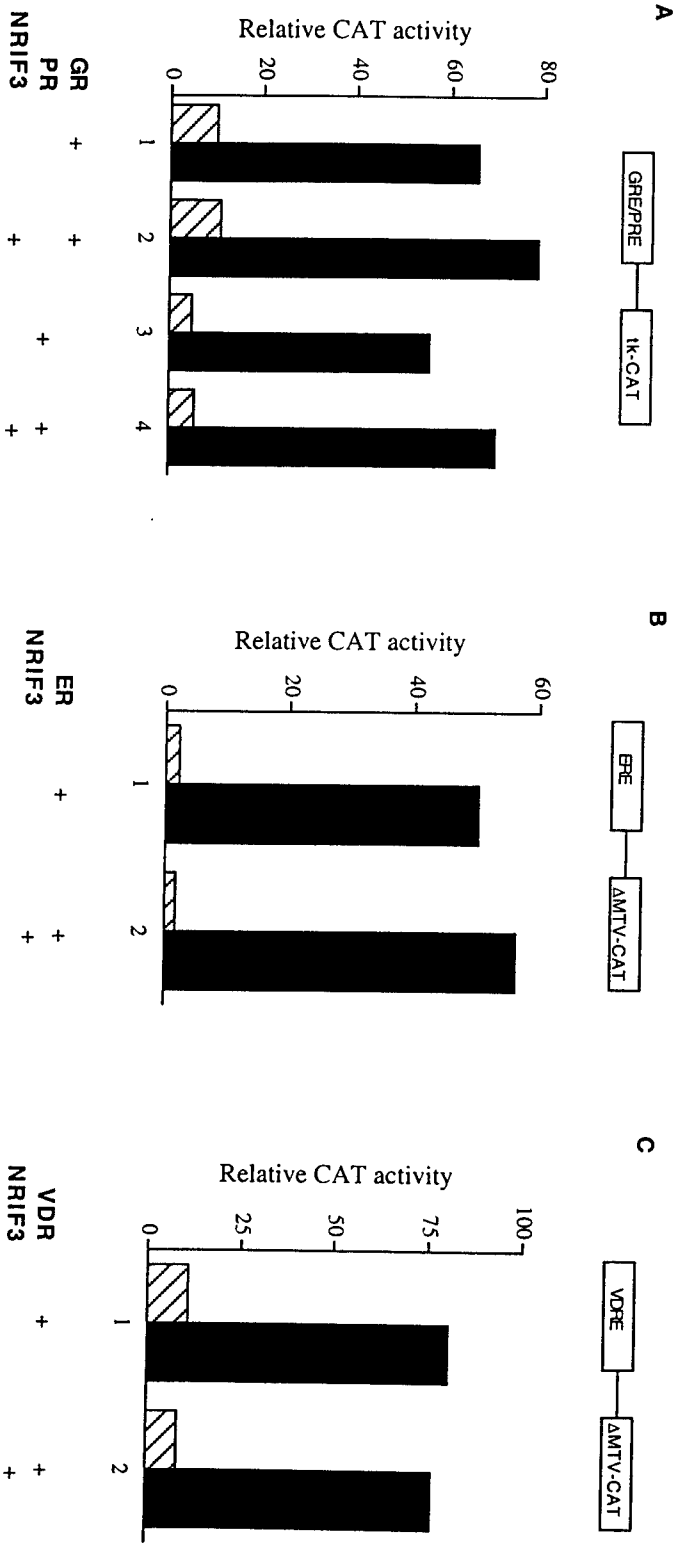
FIGURE 7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ERE
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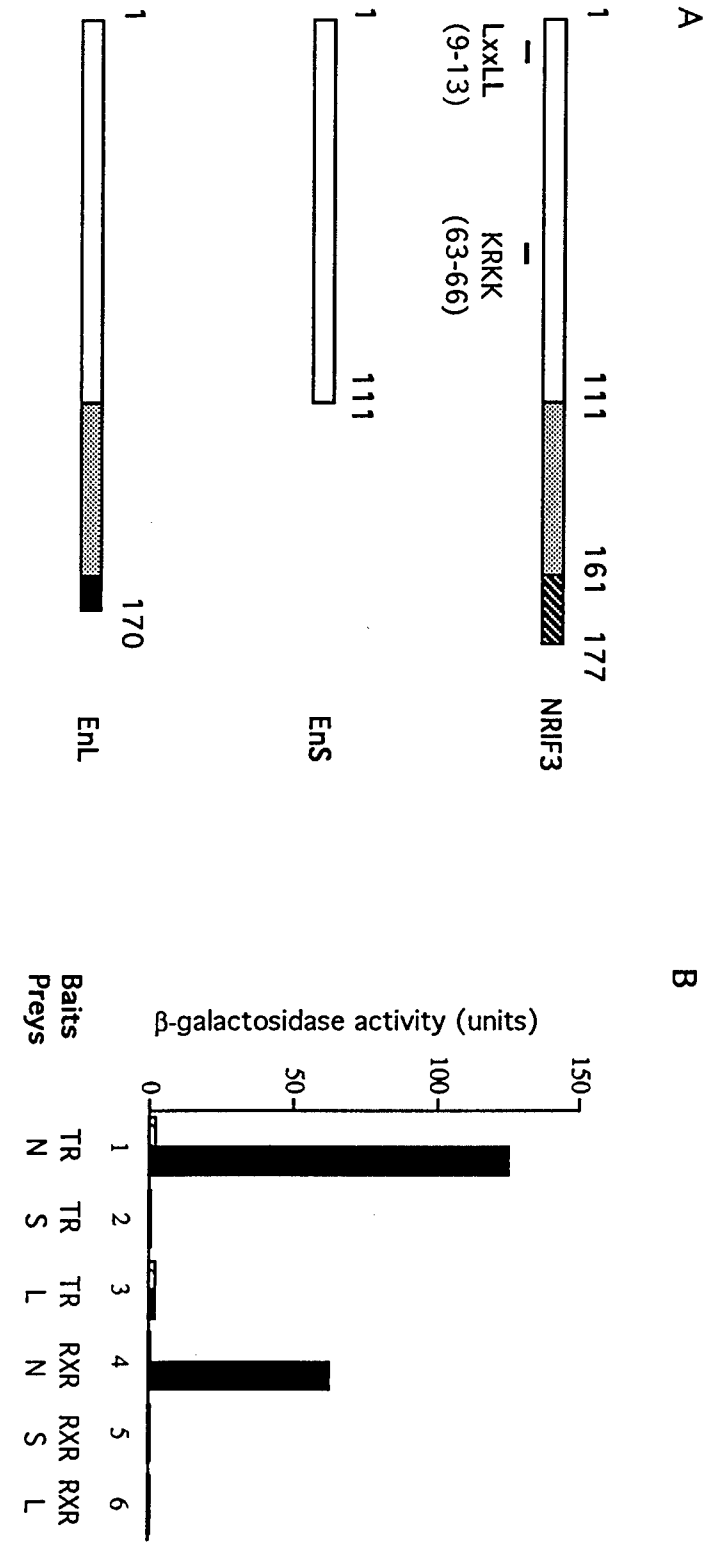
ΔMTV-CAT

FIGURE 8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EnL
B
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50
100
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TR L
RXR N
RXR S
RXR L
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2
3
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6

FIGURE 9

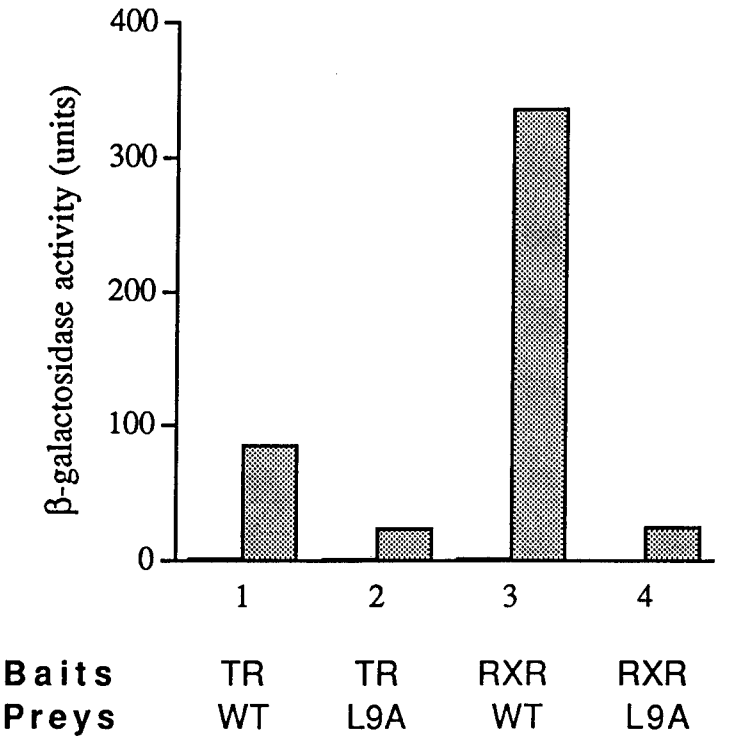
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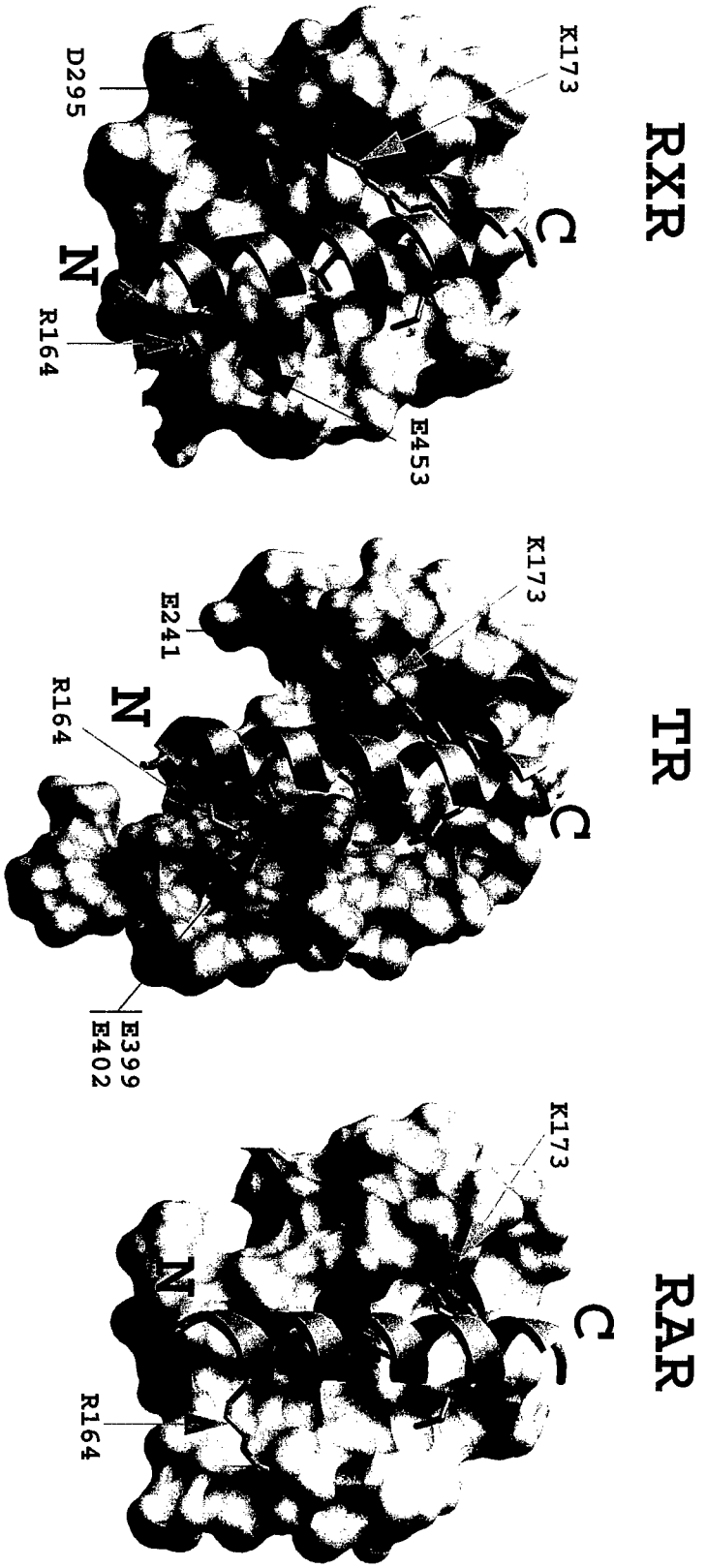
FIGURE 10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K173
C
N
R164

**Rational design of novel Nuclear Hormone Receptor Antagonists**

Matthieu Schapira, Bruce Raaka, Herb Samuels, and Ruben Abagyan

**Abstract**

Nuclear Hormone Receptors (NR) are potential therapeutic targets for many diseases, including cancer, diabetes and deregulation of brain functions. The crystal structure of the ligand binding domain of several active NR enables the design of agonists. However, with the exception of the estrogen receptor, the lack of inactive receptor structure made the rational design of antagonists impossible. In this work, we present a strategy to design such molecules: we build a model of the inactive conformation of Retinoic Acid Receptor, and use it to discover novel antagonists. The currently available crystal structures of NR can therefore be sufficient for the rational design of antagonists, which could lead to the development of novel drugs for a variety of diseases.

## Introduction

The Nuclear Hormone Receptors (NR) are ligand regulated transcription factors. Members of the family are under the control of a wide variety of hormones, such as steroids, retinoids, the thyroid hormone, vitamin D or prostanoids, and many of them are potential targets for the therapy of a variety of diseases (Table 1 for references): the estrogen receptor is the receptor for tamoxyfen in breast cancer therapy, Retinoic Acid Receptor (RAR) ligands have been shown to prevent growth of certain cancer cell types and are undergoing clinical trial, Retinoid X Receptor (RXR) and Peroxysome Proliferator-Activated Receptor γ (PPARγ) ligands could be good candidates against cancer and diabetes (PPARγ is the receptor for the antidiabetic drug thiazolidinedione), Nurr1 ligands could be useful for treatment of Parkinson's disease. Designing molecules able to activate or inhibit specific NR is therefore a challenge of high medical relevance.

The crystal structure of the Ligand Binding Domain (LBD) of many members of the family has been solved during the past years, and the structural mechanism of activation is understood in great details (Moras98 for review): upon binding of an agonist, the C-terminal domain of the LBD undergoes a conformational change and closes like a lid on the ligand, thereby generating a hydrophobic pocket at the surface of the receptor. This pocket is the target for coactivator proteins which bind the LBD through a conserved LxxLL motif (Feng98, Nolte98, Darimont98). The wealth of structural information concerning this activation mechanism has made possible the design of agonists for many NR (Klaholz98, Moras98). However, until recently, nothing was known about the structure of the LBD bound to an antagonist. Two recent papers shed some light on this critical aspect of the regulation of NR metabolic pathways: the structure of the estrogen receptor LBD bound to tamoxifen and raloxifen, two antagonists, revealed at the atomic level the characteristics of the inactivated form of a receptor of the family (Brzozowski97, Shiau98). Moreover, the observation that antagonists for other NR, such as RU486 for the progesterone receptor, share some structural features with these two antagonists, suggested that the structural mechanism of receptor inactivation by antagonists could be conserved among the NR (Cadepond97, Williams98). Our goal in this work is to present a proof of concept for this hypothesis,

build a model of the antagonist-bound conformation of a member of the family unrelated to the estrogen receptor, and rationally design antagonists for this receptor.

The retinoid receptors have a critical role in the differenciation and proliferation of wide variety of cell types (Fitzgerald97, Giannini97, Chao97 ). Retinoic Acid Receptor (RAR) agonists were originally recognized as potential anticancer agents (Lippman87, Lippman92, Dawson95). However, serious side effects were observed and the results in the clinic were not entirely satisfactory (Hong90, Sutton97, Budd98). RAR antagonists were also recently demonstrated to be promising molecules against breast cancer (Toma98, Fanjul98), mayby through cooperation with the Retinoid X Receptor (RXR) (Westin98). The observation that RAR antagonists could induce apoptosis of both estrogen receptor positive and negative cells, and the apparent absence of undesired side effects make these compounds particularly interesting (Fanjul98). The structure of the RAR LBD bound to the natural agonist all-trans Retinoic Acid (all-trans RA) is known, but there is no information on the antagonist-bound conformation. The RAR is therefore a medically and conceptually relevant target for the design of NR antagonists, without direct information on the structure of the ligand binding site.

In the present work, we built a model of the antagonist-bound structure of RAR, based on the estrogen receptor/tamoxifen complex. The docking of two known antagonists (AGN193109 and MX781) into the modeled pocket produced excellent fit, a first indication of the validity of our model. The model was then used for the virtual screening of a database of 150000 available molecular compounds, and antagonist candidates were tested for repression of expression of a reporter gene, using an RAR-Gal4 fusion construct. Two novel antagonists and a novel agonist were discovered. The ligands were specific for RAR, confirming the validity of our model, and the medical potential of our strategy.

**Modeling of the RAR antagonist binding pocket**

The X-ray structure of RARγ bound to the agonist all-trans RA is available (Renaud95), however, the conformation of the receptor bound to an antagonist is not known. A first step was therefore to build a model of this complex. We used observations

made from the structure of estrogen receptor bound to an agonist, 17-β-estradiol, on one hand, and two antagonists, tamoxifen and raloxifen, on the other hand, to build this model (Brzozowski97, Shiau98).

First, agonists and antagonists superimpose well, and engage in a very similar network of hydrophobic and electrostatic contacts with the receptor. However, in the agonist-bound conformation, the C-terminal helix H12 sits like a lid on top of the ligand (Brzozowski97) (a similar observation was made for virtually all of the NR LBD structures solved so far (Moras98 for review)). On the other hand, the two antagonists present a protruding arm which is not compatible with the "closed lid" conformation (Brzozowski97, Shiau98) (Fig. 1A). As a result, helix H12 is pushed away from the hormone binding site.

Second, the two structures of the estrogen receptor bound to antagonists reveal the new localization for the C-terminal helix: it interacts with a hydrophobic groove at the surface of the receptor which coincides with the binding site of coactivator proteins, necessary for transactivation (Fig. 1B). Of particular interest is the observation that the LxxML motif of helix H12 mimics, and probably competes with, an LxxLL helical peptide of the coactivators, the signature of the members of this family. The alignment of the NR LBD (Wurtz96) shows that the helix H12 ZxxZZ motif (where Z is a hydrophobic residue) is conserved throughout the different members of the family, suggesting that it could compete with the coactivator LxxLL motif for the other NR as well. A common structural mechanism would be for the antagonists to induce the relocation of helix H12 into the hydrophobic coactivator-binding groove of the receptor. The observation that the progesterone receptor antagonist RU486 superimposes with the natural hormone progesterone, but presents a protruding arm very similar to the one of tamoxifen (Cadepond97, Williams98) is in favor of the universality of this mechanism of antagonistic activity.

To build our model, we docked helix H12 of RARγ into the putative coactivator binding pocket of the receptor as described elsewhere (Li99) (Fig.1C), and remodeled the 25 C-terminal residues, starting at the end of helix 11, through an extensive global energy minimization procedure (Fig.1D).

## Docking of known antagonists into the modeled receptor

A few RAR antagonists have been described in the literature, some of which are serious candidates for cancer therapy (Toma98, Fanjul98). A well characterized ligand is AGN193109, which can bind and inhibit the 3 isoforms of RAR (RARα, RARβ and RARγ) at nanomolar concentrations (Johnson95). Another very potent antagonist recently reported is MX781, which is effective against estrogen receptor-positive and –negative breast cancer cells, with no apparent toxicity (Fanjul98). The activity of these two ligands has been presented in detail, but no structural information was reported on their mode of interaction with the receptor. We built a model of RARγ complexed either to AGN193109, or to MX781, using our flexible docking algorithm (totrov97) (Fig.2A and B). In both cases, the antagonist superimposed perfectly with the agonist all-trans RA. As observed for the estrogen receptor, the antagonists also presented a protruding arm, which was absent in agonists. Very importantly, this protruding arm coincided exactly with the single opening in the ligand binding pocket of our modeled receptor, generated by the displacement of helix H12 (Fig. 2A and B), and made stabilizing hydrophobic contacts with the protein. It is very unlikely that this perfect fit, observed for both antagonists, was fortuitous. On the contrary, this feature mimics the inactivation mechanism revealed by the crystal structure of estrogen receptor bound to tamoxifen and raloxifen. Therefore, the docking of AGN193109 and MX781 suggested very strongly that: 1) Our working hypothesis was true: the mechanism for antagonizing the estrogen receptor is transferable to other NRs. 2) Our model of the RAR antagonist binding pocket could now be used to design novel antagonists.

## Screening of a virtual library and discovery of novel RAR antagonists

High throughput screening is today the most utilized method for the discovery of receptor specific ligands. This is a very efficient method, but it is also very heavy, requiring the physical availability and management of hundreds of thousands of chemical compounds. In the present work, we used a virtual library, composed of the structure (generated from their smiles sequence) of over 150000 available compounds. Each

compound was automatically docked in a grid representation of the RARα binding pocket (the RARα binding pocket was derived from our model of RARγ, where the 3 non-identical residues in the vicinity of the binding pocket –A234, M272, and A397- were mutated to the RARα isoform –S234, I272 and V397-, and energy-minimized). Five grid potentials carried information on the shape, hydrophobicity, electrostatics, and hydrogen-bonding availability of the receptor, and enabled a rapid and accurate docking simulation (Totrov99?).

153000 compounds were docked automatically to the RARα antagonist binding pocket. RARα was selected over the other two isoforms because recent data suggests it could be a medically more relevant target (Toma et al.). After an automatic selection procedure with flexible ligands, and optimization of the selected candidates with flexible protein side-chains (see Methods for details), 32 compounds were considered as potential antagonists of RARα and ordered.

To test these compounds *in vitro*, an RARα-LBD/Gal4 fusion construct was activated by the natural hormone all-trans RA, which induced the expression of a chloramphenicol acetyltransferase (CAT) reporter gene. The CAT activity was then measured, as each antagonist candidate was added to the solution. The toxicity of the compounds was deduced from the amount of cellular protein extract after 3 days. Two antagonist candidates inhibited the CAT activity by 50% at 10μM with no toxicity (Fig. 3). No inhibition was observed when CAT expression was under the control of an RXR-LBD/Gal4 fusion construct, showing that: 1) the antagonists are specific for RAR 2) the inhibition is due to interaction with the fusion construct, and not CAT (data not shown).

The two RARα antagonists dock into the ligand binding pocket of the receptor (Fig. 2C and D). As observed for AGN193109 and MX781, they superimpose well with the natural agonist all-trans RA, but present an additional protruding arm, which sticks out of the pocket. Antagonist 1 has a tri-fluoro group where the retinoid receptor ligands usually carry a carboxylate group (in Antagonist 2, the corresponding domain is troncated). An obvious way to increase the affinity of these antagonists would be to substitute the tri-fluoro group by a carboxylate in Antagonist 1, or elongate and add a carboxyalte in Antagonist2, which would engage in more stabilizing interactions with two conserved arginines of the receptor. However, the purpose of this work is to show it

is possible to rationally design antagonists from the model of the inactive receptor, and not to optimize the affinity of the compounds. The *in vitro* essays show that our modeling scheme is relevant, and can be used to design novel antagonists of NR.

**Discovery of novel RARα agonists**

The 32 RARα antagonist candidates were also tested experimentally for RARα agonist activity (data not shown). One of them, Agonist 1, induced activation of the CAT reporter gene at 500 nM (data not shown). This result was unexpected and interesting. Agonist 1 is much bigger than all-trans RA, which explains why it was selected as a potential antagonist (Fig. 4A and B). However, the docking of Agonist 1 into the binding pocket of active RARα shows that it can adopt a conformation where it fits very tightly into the receptor LBD and superimposes reasonably with all-trans RA (Fig 4B, right), making more extensive hydrophobic contacts with the receptor, which could explain its high affinity for RARα. Agonist 1 also has a pyridine group instead of the conserved carboxylate, and is very different from all retinoid receptor agonists described so far: it could be a very good starting point to develop novel high affinity RAR agonists.

To complete our study, we screened the virtual library for molecules which fitted into the binding pocket of the active form of RAR (the crystal structure of RARγ was used as the receptor). 30 agonist candidates were tested *in vitro* for their ability to induce CAT activity. Two compounds, Agonist 2 and Agonist 3, induced CAT activity at 1μM concentration (Fig. 4C and D). Both of these compounds were novel agonists, had the carboxylate common to all RAR ligands described in the literature, and superimposed well with all-trans RA. Agonist 1 was RAR specific, and did not activate an RXR-GAL4 fusion construct at concentrations tested (up to 20μM, data not shown), but Agonist 2 and 3 did activate both RAR and RXR.

**Discussion**

In this study, we presented a new strategy to discover antagonists, as well as agonists, for Nuclear Hormone Receptors, very important targets for drug design (Table 1).

An important aspect of our approach was to exclude any preconceived pharmacophore bias from our database screening. Most drug design strategies are imposing chemical constraints on the selected molecule, in order to conserve the functional groups believed to be most important in existing ligands, preventing the discovery of novel ligand types. In the present work, we avoided pharmacophore constraints due to a robust flexible docking program and scoring function: the only filters used for screening were a good fit with the receptor, and reasonable bioavailability parameters (Lipinski97). As a result, we discovered ligands with unexpected chemical and structural features, such as replacement of a conserved carboxylate by a nitro (Antagonist 2, Fig.2D) or a pyridine group (Agonist 1, Fig.4B ), and unusual size (Agonist 1, Fig 4B). These novel RAR ligands present therefore valuable information for the design or optimization of RAR selective antagonists and agonists, which could have important biomedical applications.

Another important point was to demonstrate that we could discover novel antagonists for a nuclear receptor unrelated to the estrogen receptor, provided the structure of the active form of the protein was known. The retinoic acid receptor was a relevant and distant example, since, unlike the estrogen receptor, it does not belong to the steroid receptor family. Rational design of ligands from a model of the receptor is still considered today with skepticism by most professionals in the drug discovery field, and expected success rates are very low. The present study demonstrates that this strategy can be successfully undertaken with appropriate biological systems, and robust modeling tools. Moreover, targeting models of diverse members of the NR family could be further justified by the wealth of structural and sequence information (Wurtz96, Moras98), as well as a belief that the NR family members share the same mechanism of activation/inactivation (Moras98).

The recent publication of the crystal structure of medically relevant protein targets, such as PPARγ (Nolte98), RAR (Renaud95), RXR (Bourguet95), estrogen receptor (Brzozowski98) or progesterone receptor (Williams98), has created an exiting

opportunity for the discovery of novel inhibitors. This study demonstrates that the rational design of both antagonists and agonists, using computer generated models based on these structures, is possible.

## Methods

### Building of the model of antagonist-bound RAR

The helical peptide PLIREMLENP corresponding to helix H12 of RARγ was docked into the putative coactivator binding pocket of another RARγ molecule, as described elsewhere (li99). We hypothesized that the IxxML motif contacts the coactivator binding site, and an automatic docking procedure was carried out towards this site, with flexible protein and peptide side-chains, according to a Biased Probability Monte Carlo energy minimization procedure (Totrov94, Strynadka96). Tethers were then set between the C-terminus of the receptor and the corresponding docked helix, and the energy of the 25 C-terminal residues was minimized by a Monte Carlo sampling of the conformational space, with internal coordinates (Abagyan94).

### Receptor-ligand docking

An initial docking was carried out with a grid potential representation of the receptor, and flexible ligand (Totrov97). The resulting conformation was then optimized with a full atom representation of the receptor, flexible receptor side-chains, and flexible ligand, by a double-scheme Monte Carlo energy minimization procedure (Totrov97).

### Screening of a virtual library of compounds

The flexible-ligand/grid-potential-receptor docking algorithm was carried out automatically on a library of 150000 available chemical compounds. Each compound was assigned a score, according to its fit with the receptor, which took into account continuum as well as discreet electrostatics, hydrophobicity and entropy parameters (Totrov99). The best scoring compounds were further minimized with a full atom representation of the receptor, as described above.

**Acknowledgments**

This research was supported by DOD grant DAMD179818133, NIH grant GM5541801 and DOE grant DEFG0296ER62268 to RA and MS.

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TABLE 1

| Target Receptor | Disease | References |
|---|---|---|
| RAR | Breast, Cervical Cancer<br>Lung Cancer<br>Leukemia | budd98,fanjul98,Lippm87, Lippm92<br>Zhang96,Li98<br>Shiohara99,lococo98,tallman98 |
| RXR | Breast , Ovarian Cancer<br>Lung, Prostate Cancer<br>Leukemia<br>Diabetes, Obesity | bischoff98,miller97,wu97,Chao97<br>Li98, deVos97<br>Shiohara99,lococo98,tallman98<br>mukherjee97 |
| PPARγ | Diabetes, Obesity<br>Breast, Prostate Cancer<br>Colon Cancer | forman95,saltiel96,spiegelman98,deeb98<br>Elstner98,mueller98,kubota98<br>sarraf98 |
| ER | Breast Cancer<br>Infertility | dees98<br>kregge98,hess97,korach94 |
| Vitamin D Receptor | Osteoporosis | keen97,eisman96 |
| Androgen Receptor | Prostate Cancer | ross98,culig98 |
| Nurr1 | Parkinson's Disease | zetterstrom97 |

FIGURE 1

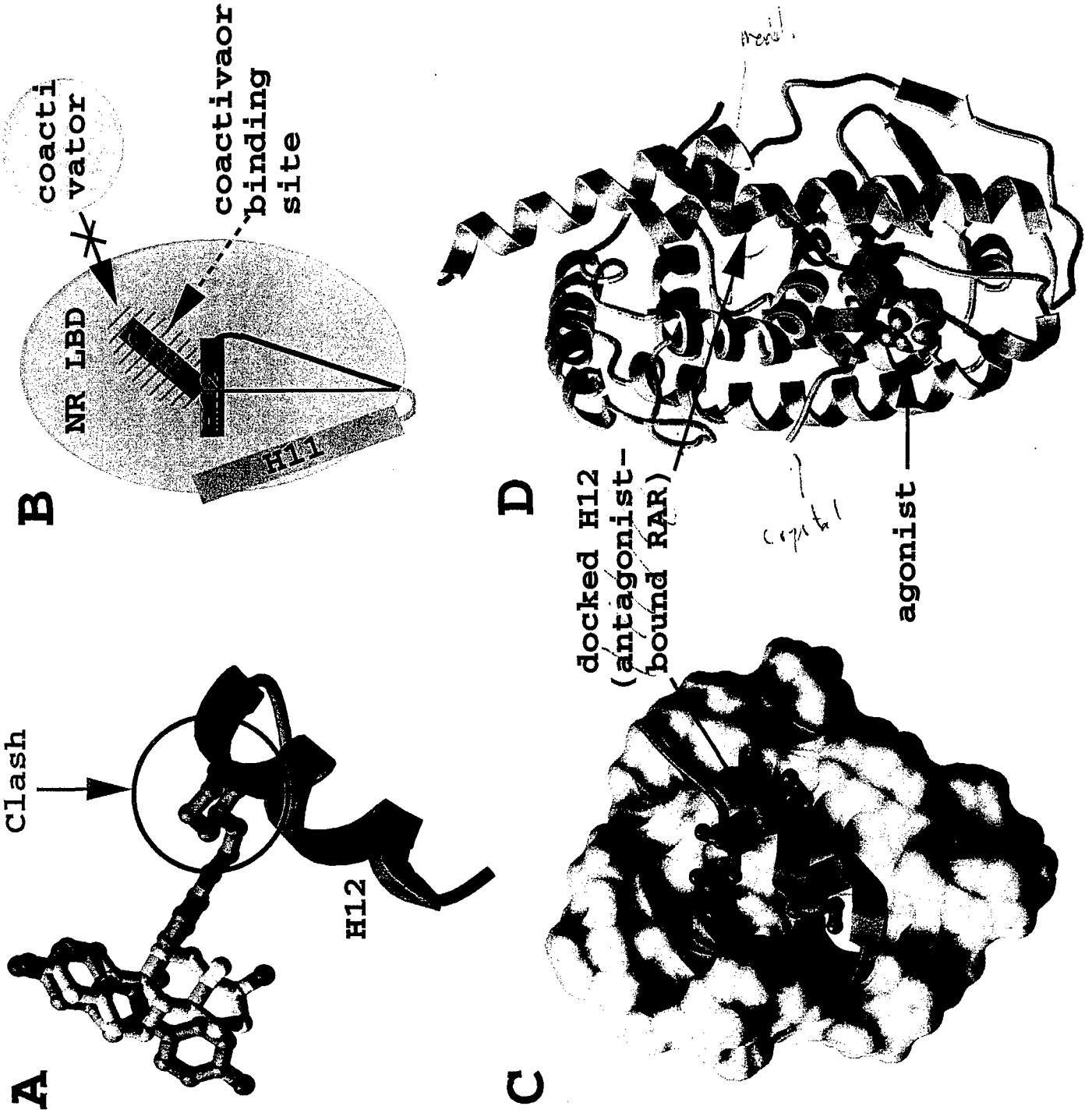
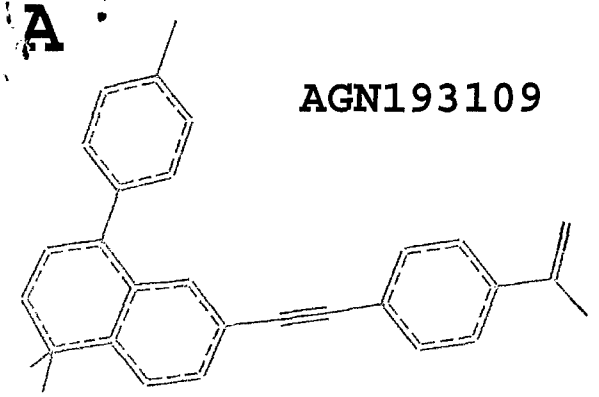
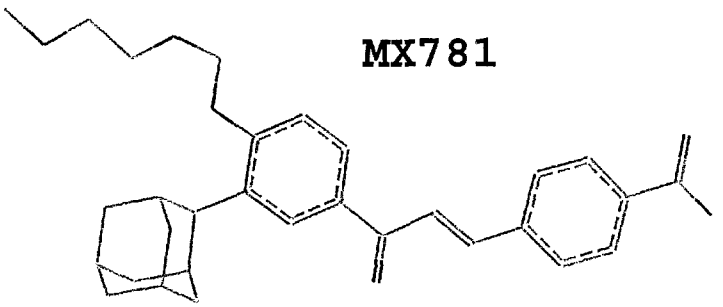

A
Clash
H12
B
coacti
vator
coactivaor
binding
site
NR LBD
H11
C
D
docked H12
(antagonist-
bound RAR)
agonist

FIGURE 2

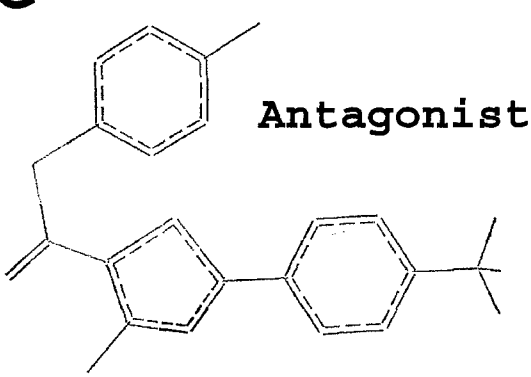
A

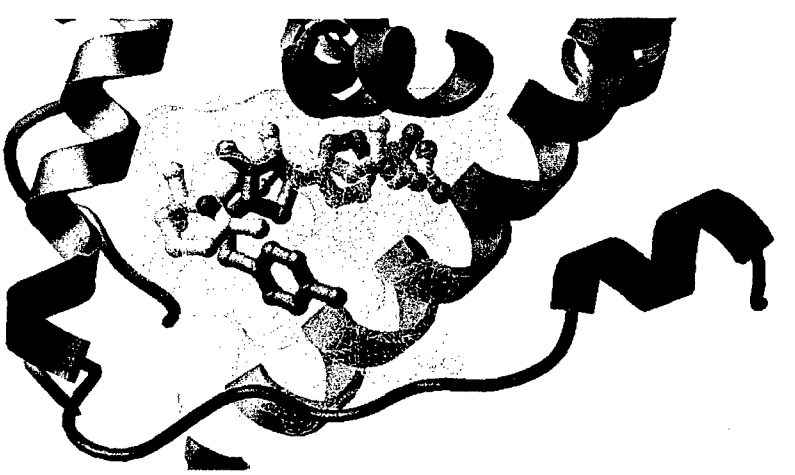



B




C



D

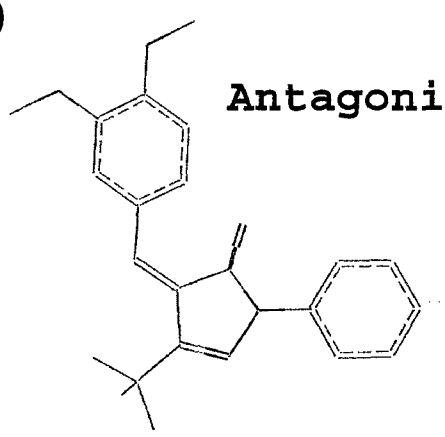



FIGURE 3

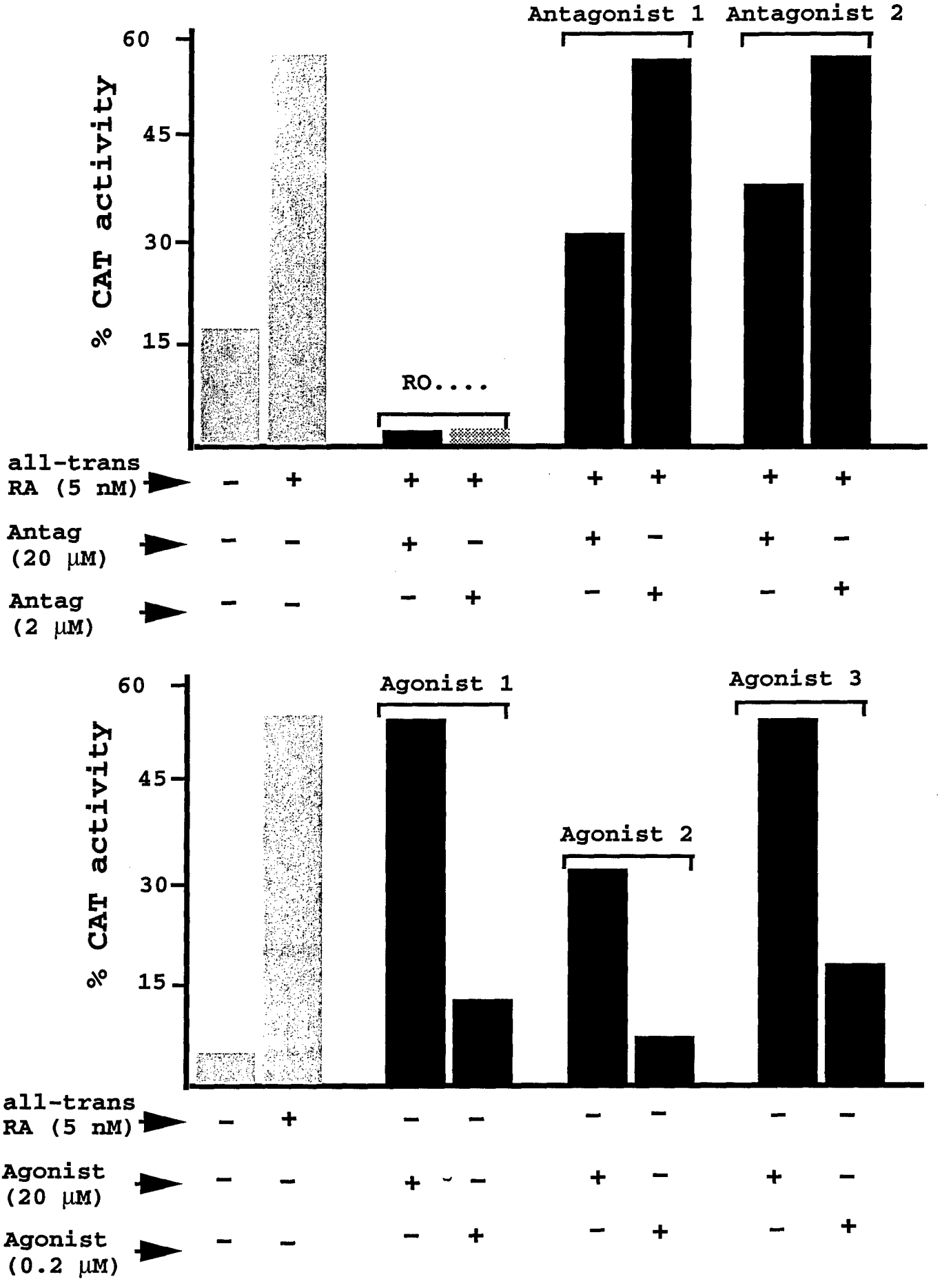
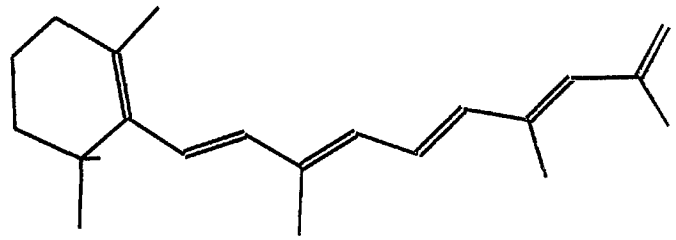

Antagonist 1
Antagonist 2
60
45
30
15
% CAT activity
RO....
all-trans RA (5 nM)
Antag (20 μM)
Antag (2 μM)
Agonist 1
Agonist 3
Agonist 2
Agonist (20 μM)
Agonist (0.2 μM)

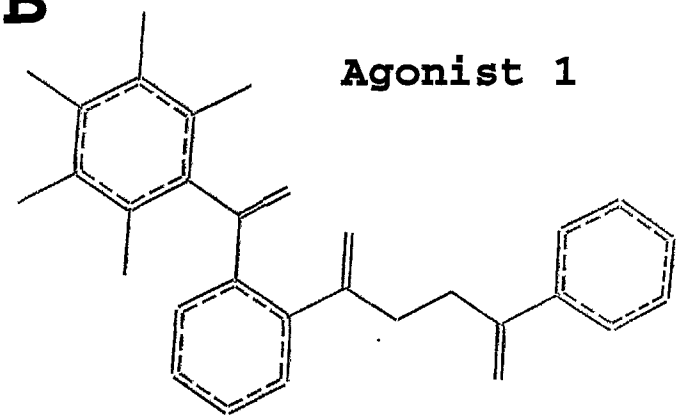
FIGURE 4

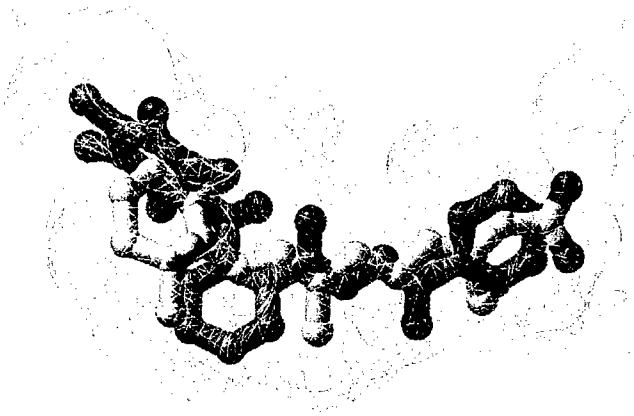
A



B

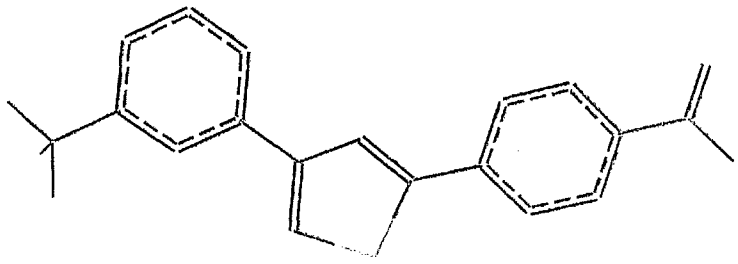
Agonist 1

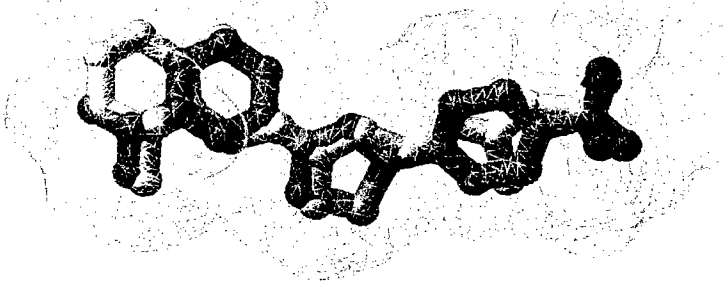




C

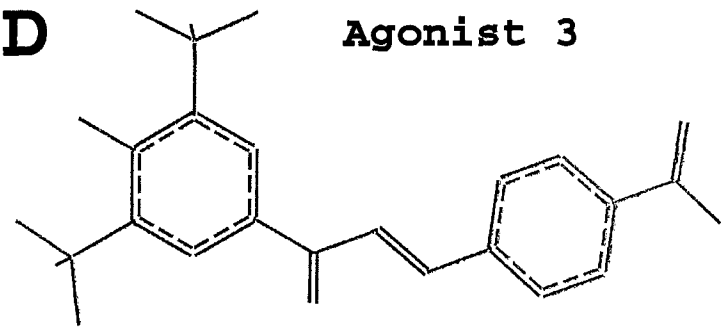
Agonist 2

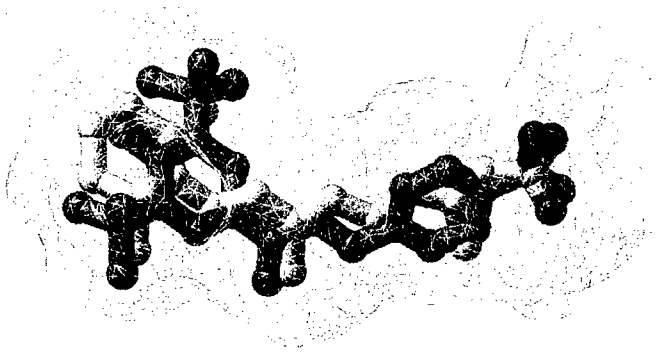




D

Agonist 3





MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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